307209
43
1995

Acta Veterinaria Hungarica

VOLUME 43, NUMBER 1, 1995

EDITOR–IN–CHIEF
J. MÉSZÁROS

ASSOCIATE EDITOR
A. SZÉKELY

EDITORIAL BOARD
A. BARTHA, J. BOKORI, T. KASSAI, F. KUTAS,
G. PETHES, P. RUDAS, L. SOLTI, S. TUBOLY,
J. VARGA, F. VETÉSI. K. VÖRÖS

Akadémiai Kiadó, Budapest
ACTA VET. HUNG. 43 (1) 1—191 (1995) HU ISSN 0236–6290

ACTA VETERINARIA

A QUARTERLY OF THE HUNGARIAN ACADEMY OF SCIENCES

Acta Veterinaria publishes reviews and original papers on veterinary science in English.

Acta Veterinaria is published in yearly volumes of four issues by

AKADÉMIAI KIADÓ

Publishing House of the Hungarian Academy of Sciences

H–1117 Budapest, Prielle Kornélia u. 19—35.

Manuscripts and editorial correspondence should be addressed to

Acta Veterinaria

Veterinary Medical Research Institute of the
Hungarian Academy of Sciences

H–1581 Budapest, P.O. Box 18

Subscription information

Orders should be addressed to

AKADÉMIAI KIADÓ

H–1519 Budapest, P.O. Box 245

Subscription price for Volume 43 (1995) in 4 issues US$ 96, including normal postage, airmail delivery US$ 20.00.

Acta Veterinaria is indexed in Current Contents

Acta Veterinaria Hungarica is abstracted/indexed in AGRICOLA, Biological Abstracts, Bibliography of Agriculture, Chemical Abstracts, Current Awareness in Biological Sciences, Current Contents–Agriculture, Biology and Environmental Sciences, Dairy Science Abstracts, Excerpta Medica, Focus On: Veterinary Science and Medicine, Helminthological Abstracts, Index Medicus, Index Veterinarius, International Bibliography of Periodical Literature, La Presse Médicale, Nutrition Abstracts and Reviews, Review of Plant Pathology, Soils and Fertilizers, Veterinary Bulletin.

"This periodical is included in the document delivery program THE GENUINE ARTICLE of the Institute of Scientific Information, Philadelphia. The articles published in the periodical are available through *The Genuine Article* at the Institute for Scientific Information, 3501 Market Street, Philadelphia PA 19104."

© Akadémiai Kiadó, Budapest 1995

CONTENTS

Animal nutrition

Complex study of the physiological role of cadmium I. Cadmium and its physiological role. *Bokori, J.* and *Fekete, S.* 3

Complex study of the physiological role of cadmium II. Effect of cadmium load on the cadmium content of eggs. *Bokori, J., Fekete, S., Kádár, I.* and *Albert, M.* 45

Animal reproduction

In vitro and *in vivo* motility studies of ^{99m}Tc HM-PAO labelled sperm cells. *Balogh, L., Szász, F., Zöldág, L., Huszenicza, Gy., Tóth, L., Dabasi, Gabriella* and *Jánoki, Gy.* 63

Effects of sample handling temperatures on bovine skim milk progesterone concentrations. *Eissa, H. M., Nachreiner, R. F.* and *Refsal, K. R.* 79

Effect of luteinizing hormone and estradiol on *in vitro* maturation of follicular oocytes in goat (*Capra hircus*). *Sidhu, K. S.* and *Cheema, S.* 89

Bacteriology

The production of K88 antigen by *Escherichia coli* and *Salmonella typhimurium* strains with recombinant DNA. *Holoda, E.* and *Mikula, I.* 95

Immunology

Intestinal absorption of colostral lymphocytes in newborn lambs and their role in the development of immune status. *Tuboly, S., Bernáth, S., Glávits, R., Kovács, Andrea* and *Megyeri, Z.* 105

Mycotoxin research

Contamination of broiler chicken's mash and litter with moulds, aflatoxins, ochratoxin A and zearalenone. *Škrinjar, M., Ristić, M.* and *Grbić, Z.* 117

Parasitology

Histological changes in the swimbladder wall of eels due to abnormal location of adults and second stage larvae of *Anguillicola crassus. Molnár, K., Szakolczai, J.* and *Vetési, F.* 125

Ultrastructural observations on the third-generation merozoites of *Eimeria tenella* in chicks. *Ball, S. J., Daszak, P., Pittilo, R. M.* and *Norton, C. C.* 139

MAGYAR
TUDOMÁNYOS AKADÉMIA
KÖNYVTÁRA

Pathology

Eosinophils in lymph nodes of cows infected by bovine leukaemia virus. *Levkut, M., Levkutová, Maria, Konrád, V.* and *Poláček, M.* 145
Effect of mercury on the seminiferous epithelium of the fowl testis. *Maretta, M., Marettová, E., Škrobánek, P.* and *Ledeč, M.* 153

Physiology

Variations in the milk yield and milk composition of dairy cows during lactation. *Bedő, S., Nikodémusz, Etelka, Percsich, K.* and *Bárdos, L.* 163
Effect of severe energy restriction and refeeding on thyroid hormones in bulls. *Janan, J., Rudas, P., Bartha, T., Bozó, S.* and *Gábor, Gy.* 173
Effect of hydrothermal treatment of rice straw on its composition, *in sacco* digestibility and *in vitro* fermentation by rumen microorganisms. *Adya, M., Sareen, V. K.* and *Singh, Sudarshan* 179

Coming events 191

Acta Veterinaria Hungarica 43 (1), pp. 3–43 (1995)

COMPLEX STUDY OF THE PHYSIOLOGICAL ROLE OF CADMIUM I. CADMIUM AND ITS PHYSIOLOGICAL ROLE

J. BOKORI and S. FEKETE

Department of Animal Nutrition, University of Veterinary Science, H–1400 Budapest, P.O. Box 2, Hungary

(Received March 16, 1994)

It has been known for more than 70 years that cadmium (Cd), an element ubiquitous in nature, gets into the organism of both plants and animals and accumulates in their organs. Over a long period, only the deleterious effects of Cd were reported; however, in recent years the adverse consequences of even a minor Cd deficiency have been demonstrated in animal experiments. In spite of this, it has not been declared essential to date (Anke et al., 1977; Schwarz and Spallholz, 1979).

Elemental Cd is a silvery-white metal which shares a group with Zn and Hg in the periodic system. In a dry environment it is stable, while in a humid environment and at high temperature it is slowly oxidized and changes into reddish-yellow cadmium oxide of unpleasant odour. Cd is bivalent in its compounds. It is more soluble in acidic medium. Its oxides and sulphates are involved in air pollution. Of the colourless salts of Cd, its chloride, sulphate and nitrate dissolve fairly well, while its sulphide and oxide dissolve only in acids.

1. The occurrence of cadmium

1.1. The occurrence of cadmium in nature

Cadmium constitutes 0.000011% of the *earth's crust*. It occurs in larger quantities (1 g/kg) in zinc ore and in smaller amounts (20–2,000 mg/kg) in copper and lead ore. As its melting point (312 °C) is lower than that of the Zn- and Cu-containing ore, during the treatment of the latter types of ore, as well as during glass-making, porcelain and paint production substantial amounts of Cd are released and cause environmental pollution (Liebig, 1913). The concentration of Cd in the European agricultural soils is 0.5–1.0 mg/kg; however, in heavily polluted soils (e.g. in Japan) its concentration may reach 69 mg per kg of soil (Friberg et al., 1979*a, b*). The highest amount of Cd is contained by argillaceous soils. According to surveys conducted in Hungary (Régius-Mőcsényi, 1991), plants

0236–6290/95/$ 4.00 ©1995 *Akadémiai Kiadó, Budapest*

grown on alkali soils contained the highest amount of Cd, followed by plants grown on loess, marshy and calciferous sandy soils. The Cd content of alluvial and Triassic soils was nearly identical (Table 1). The data presented in Table 2 show that different agrotechnical methods (e.g. fertilization) and environmental factors (degree of industrialization) may give rise to highly variable degrees of Cd pollution. The Cd occurring in the soil in different forms (dissolved, colloid, organically bound Cd) is rather immobile and is mostly retained in the cultivated upper layer of the soil where it may accumulate.

Table 1

Average soil-specific cadmium content of indicator plants (Régius-Mőcsényi, 1991)

Soil type	Average Cd content (μg/kg dry matter)	s	%
Sodic soil	26.5	19.8	100
Loess soil	25.5	11.0	96
Trias detrital soil	23.5	12.5	89
Peaty boggy soil	23.3	13.5	88
Diluvial sandy soil	23.3	20.0	88
Soddy-alluvial soil	23.0	12.6	87
Andesite detrital soil	21.8	13.0	82
Acidic sandy soil	21.3	10.5	80

Freshwaters contain small amounts of Cd of a widely varying concentration (0.002–0.15 mg/l). Fresh tap-water contains 1 μg/litre (Fleischer et al., 1974*a, b*) or 1.3 μg/litre (Ryan et al., 1982). This means that approximately 3 μg Cd is ingested if we assume a daily water intake of 2 litres. Larger amounts of Cd occur in the water of rivers, partly in dissolved form, partly in the mud (Bokori, 1994). For instance, the waste-water of galvanizing plants may contain as much as 100–500 mg Cd per litre. The water of oceans contains much Cd, primarily in regions close to industrial areas.

The *air* contains low amounts of Cd: its average Cd concentration is 1 ng/m^3 in the country and about 20 ng/m^3 in urban areas. Much Cd is present in the air around the big volcanic mountain ranges (e.g. 30 ng/m^3 near the Etna). Large amounts of Cd are released into the air by industrial plants burning coal and oil, as well as by ore and cement processing plants: in the vicinity of such plants Cd is one of the major pollutants. According to Friberg et al. (1985), from the zinc ore necessary for the production of 1,000 t Zn approx. 3 tons of Cd are released into the environment. E.g. in Germany the total Cd pollution of the environment amounted to approx. 328 tons in 1979 (Pacyna, 1983). The size of the Cd powder

particle is around 2 μm depending on the source of emission (Godzik et al., 1979). The total amount of cadmium causing soil and plant "contamination" and a Cd load to animals is usually estimated at 1–5 g/hectare/year. In such "contaminated" areas the free-living ruminants are exposed to the heaviest Cd load (Marletta et al., 1986).

Table 2

Cadmium levels in some agricultural and urban soils (Cd in phosphates, 1989)

Location	Cadmium content (ppm)	
	Range	Average
Agricultural soils		
W. Germany	0.4–0.5	
Norway	0.01–0.64	
Denmark	0.03–0.9	
Japan	<1	0.3
Canada	0.55–1.80	
Sweden	0.03–2.3	
EEC	0.01–2.5	
Urban soils		
Sudbury, Ontario	0.20–2.90	
Manchester, England		3.4
Toronto		5
Trail, BC	1.60–15.00	
Noranda, Quebec		20

Due to the ever increasing industrial utilization of Cd (galvanization, electronics, plastics production, paint and fungicide production, nuclear industry), by now the annual Cd output has reached 16–20 thousand tons. Thus, environmental and soil pollution caused by Cd is the most severe in the highly developed industrial countries where the annual average increase in the Cd concentration of arable soil can be put at 1 g/hectare (Nomiyama et al., 1973). Based upon the data of global Cd emission the ecosystem is exposed to an annual average Cd load of 9.4×10^6 kg. This means that gradual pollution of the biosphere with Cd and with other toxic metals and metalloids (Hg, Pb, Ni, V, As, Se, etc.) will severely and predictably impair the health status of future generations.

Plants and primarily the green vegetative parts thereof (Kreuzer, 1986) can rather efficiently incorporate the Cd content of soil. Despite this fact, they derive a substantial part (30–60%) of their Cd content from the air, and only the rest is absorbed from the soil through the roots. The percentage proportion of Cd taken

up by plants from the air and from the soil (from rough fertilizers) is shown in Fig. 1. As the uptake of Cd from the soil is affected by many factors (e.g. by the Cd contamination of the soil, which is in a positive correlation with the Cd emission of factories), it is difficult to determine the precise route of uptake. Although the pollution does not extend to large distances, it depends on the prevailing direction of wind, the height of chimneys, and the configurations of the terrain. This is well exemplified by the Cd determinations performed in the Ruhr region (Table 3) or in the heavily industrialized Belgium where as much as 19 g of Cd may be released into the soil per hectare and per year (TECMIN: The Cadmium Question, 1984). The quality of soil, primarily its pH (Machelett and Bergmann, 1993) and its temperature markedly affect the mobilization of Cd and its incorporation into the plant (Table 4). The acidification of soils (e.g. as a result of acid rain or along roads) causes a linear increase in the Cd concentration of plants (Ahles and Rösick, 1986; Barcelo et al., 1986; Yoshida, 1986). The quantity of mobile Cd can be reduced by liming.

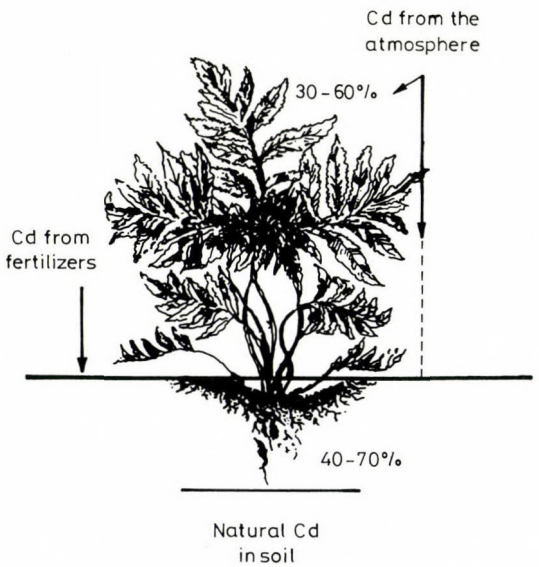


Fig. 1. Origin of the Cd content of plants (The Cadmium Question, 1984)

Table 3

Quantity of Cd deposited from the atmosphere in the industrial district (The Cadmium Question, 1984)

Surface, km^2 (year)	Deposited Cd, g/ha/year
637 (1977)	13.7
56 (1978)	27.4
3 (1979)	41.4
5 (1980)	41.4

Table 4

Effect of pH on the Cd uptake of plants (The Cadmium Question, 1984)

Plant	pH = 6.7		pH = 4.6	
	Cd supplementation, kg/ha			
	0	11	0	11
Lettuce	0.78	1.78	0.88	2.25
Cabbage	0.16	0.19	0.19	0.35
Carrot	0.71	1.25	0.96	7.29

Inorganic P fertilizers constitute an important source of soil Cd pollution (Toluczo, 1989). The Cd content of these fertilizers vary widely also depending on their origin (Table 5). The incorporation of that Cd into the plants varies between 20 and 80%, depending on the technology of fertilization and the chemical quality of the product obtained (Cadmium in Phosphates, 1989). The P preparations used for the phosphorus supplementation of feeds may contain as much as 6–7 ppm Cd.

The environmental pollution caused by superphosphate fertilizers produced in Hungary greatly depends on their method of production and on the basic material itself. Thus, crude phosphates (often cola phosphates) contain much less Cd than e.g. the North African soft phosphates; at the same time, the basic materials of rock phosphates contain large amounts of Cd, not infrequently 102 ppm. Thus, they can contribute to the accumulation of Cd, its incorporation into the food chain and the emergence of human and animal health problems related to Cd.

Much Cd may get into the soil with *sewage sludge* (Fassett, 1972; Hansen and Hinesly, 1979; Mitani and Hara, 1991) and with *animal manure*, first of all liquid manure.

Table 5

Approximate cadmium contents of commercial phosphate rocks (Cadmium in Phosphates, 1989)

Country of origin	Cadmium content (ppm)	
	Range	Mean
USSR	1	
USA (Florida)	6–15	8
Morocco	8–75	22
Israel	15–30	23
Tunisia	25–35	30
Togo	42–80	55
Senegal	66–90	75
USA (Idaho)	40–340	110

Humic soils of high organic matter content accumulate more Cd (Jones and Johnston, 1989). The Cd uptake of plants depends on ambient temperature, the oxidation-reduction potential of the soil and the concentration of other metals (Cu, Zn, Ni) taken up simultaneously (Gomez et al., 1988). E.g. Fe was found to decrease the incorporation of Cd into maize (Table 6). Cd uptake is affected also by the species (Posthuma et al., 1992) and genetic abilities of plants. Thus, much Cd is accumulated in clover (*Trifolium* spp.), in Cruciferae and in *Beta* spp., primarily in their leaves (Kreuzer et al., 1981). Many plant species have a heavy metal binding mechanism (phytoquinones of low sulphur content; Lopez and Williams, 1985) diminishing the adverse consequences of Cd load including withering, slow water transport and slow root growth (Lodenius et al., 1981).

Table 6

Antagonistic effect of iron on cadmium uptake by maize seedlings grown in nutrient solution (Cadmium in Phosphates, 1989)

Nutrient medium concentration		Cd content (dry matter basis)	
Cd mEq/l	Fe mg/l	Roots mg/kg	Aerial parts mg/kg
0.05	0	229	110
0.05	5	161	46
0.05	10	112	30
0.2	0	382	195
0.2	5	318	95
0.2	10	272	79

Favero et al. (1991) studied the Cd-incorporating capacity of the mycelia of *Pleurotus ostreatus* (Basidiomycetes) if Cu and glutathione were added to the liquid nutrient medium. Gradually increasing the concentration of glutathione up to 150 μg/ml, they found that the Cd concentration of the plant steadily decreased; however, mycelial growth did not cease completely. The most pronounced Cd accumulation (20 mg/g dry matter) was observed if Cd alone was added to the medium: in such cases almost 80% of the added Cd bound to the cell wall. The addition of Cu to the Cd-containing culture medium decreased Cd incorporation in the same way as did the simultaneous addition of Cu and glutathione. All these observations should be taken into consideration in selective plant improvement in the Cd-polluted regions.

According to extensive studies (Varo et al., 1980) the Cd content of plants grown in the same area may also vary considerably. The results of such a 5-year survey are summarized in Table 7.

Table 7

Cd concentration of cereals and vegetables based on Finnish survey (Varo et al., 1980)

Product	No. of samples	(PPB) Nanogram cadmium/gram dry matter	
		Average	Range
Grain of winter wheat ([1])	(34)	74	20–99
Grain of spring wheat ([1])	(51)	48	29–79
Grain of rye ([1])	(50)	16	5–43
Grain of barley ([1])	(47)	24	5–43
Grain of oats ([1])	(36)	48	5–79
Potato	(20)	50	20–100
Potato, fresh	(3)	150	100–300
Carrot	(5)	273	183–364
Red beet	(5)	250	83–333
Cabbage, white	(5)	63	63–125
Cauliflower	(5)	125	37–250
Lettuce	(6)	1,000	400–2,000
Spinach	(4)	2,143	714–5,000
Onion, yellow	(5)	231	39–385
Peas	(3)	125	4–292

[1]Average of five years, 1972–1976

Still, the main risk posed by Cd as an environmental pollutant is its easy access to the food chain, rather than merely its phytotoxic effect. Namely, as plants can accumulate Cd in large amounts without showing visible signs, they may eas-

ily introduce Cd into the food chain, i.e. into the soil-plant-animal-man system (Kádár, 1991).

Table 8 presents the average Cd content of some so-called indicator plants containing relatively high amounts (18–20 μg/kg dry matter) of Cd (Régius-Mőcsényi et al., 1985).

Table 8

Average cadmium content of some indicator plants
(μg/kg dry matter; Régius-Mőcsényi, 1991)

Plant species	n	x	s
Alfalfa	91	28	23
Red clover from tillage land	54	20	15
Red clover from pasture	20	29	22
Wheat	192	24	20
Rye	69	18	14

1.2. Occurrence and accumulation of Cd in human and animal nutrients

Data from France and the U.S.A. indicate that the Cd content of human foods varies widely depending on their origin. In France, 2,500 food samples were assayed, and only few of them (e.g. liver, kidney) contained Cd in excess of 100 ppb. The average Cd content measured in the majority of foods and food constituents is 10–30 ppb. The Cd content of some important food sources, as measured in Finnish studies (Varo et al., 1980), is presented in Table 7. The highest Cd concentration was measured in lettuce and spinach (average: 1,000–2,143 ppb). According to the latter authors, full avoidance of Cd-contaminated foodstuffs is impossible; therefore, only foodstuffs with Cd concentrations even higher than that (liver, kidney, certain mushroom varieties) should not be consumed over a prolonged period. As a result of environmental protection measures, the Cd content of certain moss varieties, which was steadily increasing earlier, has decreased in recent years (Gunnarsson, 1983).

The Cd content of some feed constituents is presented in Table 9. Soybean appears to contain the largest amount of Cd. Under identical Cd load the extracted groats were found to have the highest Cd concentration. The organs of free-living animals (roe-deer, red deer, wild-boar) living on soils exposed to a higher Cd load show a more expressed Cd accumulation than those of farm animals living in the same area (Kovács and Ványi, 1993).

The different parts of plants have dissimilar Cd content, in the following decreasing order: root, stalk, leaf, seed-crop. The average Cd content of plants used

for animal feeding is below 0.5 μg/g dry matter. Cereal crops contain more while leguminous plants less Cd (Baker et al., 1979).

Table 9

Cadmium content of some feed constituents in Hungary
(μg/kg dry matter; Régius-Mőcsényi, 1991)

Grain and leguminous plants Root tubes		Green feeds, silages, hays		Extr. meals, protein rich feedstuffs By-products (tillage land, industrial)	
Barley	26	Corn silage		Extracted peanut meal	120
Wheat	31	milky-waxy ripening	60	Extracted sunflower	
		waxen ripeness	55	meal	65
		complete ripeness	48	Extracted rape meal	170
Rye	43	Barley (whole plant)	52	Extracted soybean	
Oat	35			meal	29
Maize	20	Wheat (whole plant)	57	Bone-meat meal	40
Corn-cob mixture		Grass, meadow-grass		Meat meal	18
(CCM)	31	Leafing	50		
		Panicle growth	40		
		Flowering	35	Meat pulp	13
Pea	32	Summer young		Barley straw	33
Sweet lupine	300	aftermath	40	Wheat straw	28
Horse-bean	29	Summer matured after-		Autumn corn stalk	25
Potato	60	math	30	Winter corn stalk	20
Sugar beet	180	Autumn aftermath	70	Wheat bran	65
Fodder beet	201	Alfalfa	27	Sugarbeet pulp	200
		Meadow hay	49		

Juste and Mench (1992) studied the accumulation of more than 10 heavy metals in plants that had been grown on a sewage sludge treated soil for at least 10 years. In connection with Cd they made the following statements: (*i*) The seeds always contained less Cd than the vegetative parts of the plant. (*ii*) In the case of a low Cd load from sewage sludge, the seeds had higher Cd concentration even in the 6th year after the end of fertilization. (*iii*) In places (e.g. in Germany) where the Cd content of the soil approached or exceeded 2 ppm, the grain of winter wheat often had a Cd concentration of 1 mg/kg. Otherwise the maximum Cd concentration of wheat grain rarely exceeds 3 mg/kg. (*iv*) The Cd content of maize is lower than that of other cereals, probably because of the shell structure of the kernel, which constitutes a barrier to Cd incorporation. (*v*) The Cd concentration of the leaves of sugar beet grown on a soil of 5–45 mg/kg Cd content may reach

3–18 mg/kg, while the root of that plant contains only 2.5 mg/kg Cd. (*vi*) The tobacco leaf is known to be a Cd accumulating plant. In the experiment of Juste and Mench (1992), its Cd content reached 69 mg per kg of dry matter.

Wojciechowska-Mazurek et al. (1993) studied the Cd, Cu, Zn and Pb content of 500 cereal samples collected from different regions of Poland in the period 1989 through 1991. The average Cd content of wheat and rye was found to be 0.083 and 0.026 mg/kg, respectively. No major differences were demonstrated in the Cd content of cereals by region, with the exception of the southwestern industrial region, where the Cd content of cereals was slightly higher. Cd accumulates in certain fish species as well (Chaney and Hundemann, 1977).

1.3. The essentiality of cadmium

The biological role of Cd has been described by several authors from different points of view (Schroeder and Balassa, 1961; Flick et al., 1971; Fassett, 1972; Fleischer et al., 1974*a, b*).

Although under physiological conditions no Cd-activated metalloenzyme is known to exist (Morgan et al., 1985), under laboratory conditions Cd deficiency was successfully produced in experimental goats fed a semi-synthetic diet containing 20 μg Cd per kg dry matter (Anke et al., 1977, 1978). This Cd deficiency manifested itself in severely retarded growth and reproductive disturbances. Some data suggest that Cd is indispensable also for hard-shelled mollusks (Gunnarsson, 1983). In his comprehensive critical review of recent trace element studies, relying mainly on the position taken by Frieden (1985) and Horowitz (1988), Pais (1980, 1989) assigns Cd to the elements whose "physiological role has partially been proved". However, most scientist still hold the view that although Cd is known to exert beneficial physiological, biochemical and biophysical effects (Black et al., 1979; Hapke, 1992), no Cd deficiency develops under practical conditions, while Cd ingestion has severe toxic consequences (Régius-Mőcsényi et al., 1985).

2. The metabolism of cadmium

1.1. Uptake and absorption of cadmium in the human and animal organism

Cd may get into the organism from three different sources by two routes. Cd ingested with the food (feed) and drinking water through the digestive tract is relatively rapidly absorbed (6% on the average) in the duodenum and jejunum (Gruden, 1982; Rude, 1982). Absorption through the intestinal wall takes place in two steps. First the element is transported through the brush border of the mucosal epithelial cells towards the blood stream (Doyle et al., 1974; Sugawara and

Sugawara, 1975). The second step of the absorption process is slower than the first, due to the fact that the synthesis and degradation of metallothionein, the Cd-binding protein of the epithelial cell cytoplasm, can modulate the transport process in a variety of ways (Tanaka et al., 1975). In addition, the chelate-forming compounds of the intestinal content (amino acids, peptides, vitamin D, ascorbate, phytate), the metallothionein content of the detached epithelial cells, certain elements (Cu, Fe, Ca, Mg, Mn, Ni, Zn), and the pH of the intestinal content may also affect Cd absorption (Gruden and Munic, 1987; Sarhan et al., 1986). The metal chelates formed with amino acids are absorbed much more efficiently and become incorporated into the tissues in higher quantity than the ingested salts of the same metals (Ashmead and Jeppsen, 1933). Studies on Cd absorption in ruminants yielded dissimilar results. Some authors (Gründer, 1982) reported a 3–9% while others (Kreuzer, 1986) a 0.3–5% absorption rate. Xanthates and dithiocarbamates have been found to enhance Cd absorption (Block and McBladt, 1991). Kramer et al. (1993) have reported a similar effect for bentonite. The amount of Cd deposited in the organism can be effectively reduced by the administration of analogous compounds (Block and McBladt, 1991; Eybl et al., 1993).

Cd absorption is more efficient in newborn animals than in adults. Suckling animals living exclusively on milk also show a higher rate of Cd absorption (Rabar and Kostial, 1981). In animal experiments the accumulation of Cd could be largely prevented by mixing ethylenediamine-tetraacetate (EDTA) into the Cd-containing diet. EDTA forms with Cd a chelate which is excreted via the faeces; thus, a substantial part of the ingested Cd leaves the organism without causing any damage to it (May and Bulman, 1983).

Cd absorption is also affected by other elements (e.g. Zn) simultaneously present in the intestinal content (Mills, 1974). The deficiency of Ca enhances Cd absorption (Noever and Matsos, 1991). In such cases presumably the Ca-binding protein of the intestinal epithelial cells (CaBP) binds and transports Cd because of the similarity of the ionic radius of Ca and Cd (Cousins and Feldman, 1973).

Cd load reduces the motility of the intestinal wall, inhibits the intestinal absorption of transferrin-Fe, and blocks transferrin metabolism in the epithelial cells of the intestinal mucosa. As a result, Cd load may lead to anaemia even in the case of sufficient iron supply. At the same time, as Fe deficiency enhances Cd absorption and as females usually have a certain degree of iron deficiency, the Cd concentration of their organs is generally higher. There is a close correlation between Cd metabolism and the metabolism of Mg, Zn and Cu (Rambeck et al., 1991), as far as their deficiency facilitates the absorption and *in vitro* toxicity of Cd. Hoffmann et al. (1979) and Anke et al. (1982) studied Cd absorption both in mammals (e.g. in goats) and in poultry by isotope experiments. They found that e.g. in poultry nearly 10% of the orally administered Cd became absorbed in 45 min; however, only 2% of the absorbed amount was retained in the organism. In experiments with radiolabelled Cd, other authors (Berg and Kollmer, 1983) ob-

served a 0.36% absorption of Cd administered into the stomach through a tube with the drinking water. In humans the rate of Cd absorption is higher. Rahola et al. (1972) reported a 6% average absorption rate. They also demonstrated that in mammals Cd crossed the placenta, but in smaller amounts than did other trace elements.

In summary, it can be stated that Cd absorption from the intestine depends on the chemical properties of the Cd compound and the intestinal content (chemical reaction, presence of other metals). It is affected by the Fe (Fox, 1974) and Ca (Washko and Cousins, 1976) supply status of the organism, by gender (Anke et al., 1982) and by age (Bunker et al., 1984).

Substantial amounts of Cd are absorbed through the respiratory tract. The average Cd concentration of air is 0.001 μg/m^3. With the inhaled air an adult human may take up 0.03 μg Cd daily. About 10–40% of the inhaled amount will be absorbed (Friberg et al., 1974). In industrial districts, where the air may contain 0.6–10 μg Cd per m^3, as much as 100 μg Cd may get into the lungs in 15 m^3 inhaled air per day. Twenty to thirty per cent of that amount will be deposited in the lung tissue while 70–80% — according to Friberg et al. (1979*a, b*) 40% — will be absorbed.

Menden et al. (1972) and Friberg (1950) stress that the Cd pollution of air plays a major role in causing health injury. They suggest that in the immediate vicinity of smokers the Cd pollution of air (which normally contains 0.1–0.5 μg/m^3 Cd) rapidly increases and may reach a potentially harmful level. The common cigarette smoke contains 0.25–5.80 μg/g Cd. The typical average Cd content of cigarettes has been found to be 1.50 mg/kg dry matter. About 30% of the total Cd is retained in the ash and in the filter, while 70% is released with the cigarette smoke. Thus, the organism of a person smoking 20 cigarettes a day is exposed to a Cd load of 20–30 μg Cd. Moreau et al. (1983) reported a Cd content of 1.33 μg/l in the blood of active smokers and 0.37 μg/l in that of passive smokers. In Germany, the average weekly Cd intake of men and women is 0.24 and 0.18 mg, respectively (Weigert et al., 1984), which is 46 resp. 43% of the values published by the WHO (1979).

It has been confirmed in animal experiments that about half of the Cd reaching the lungs (the particles smaller than 2 μm) gets into the alveoli and is rapidly absorbed from there. At the same time, Cd deposited in the trachea and bronchi remains there for a long time, showing a half-life of 30–60 days (Nordberg et al., 1985) and, thus, slower absorption. Cd entering the organism via the lungs is approx. 60 times more toxic than that getting into it through the digestive tract (Sittig, 1976).

Table 10

Average cadmium content of some animal foods in Hungary in the years 1984 through 1988 (Sas and Tu, 1989)

Animal species	n	Cd content for total mass ($\mu mol \times kg^{-1}$)	
		x ± SD	Range
Liver			
Mammals			
Pig	974	0.872 ± 0.044	0.009–10.551
Cattle	288	0.978 ± 0.053	0.009–5.169
Sheep	60	0.267 ± 0.044	0.000–1.174
Rabbit	146	0.516 ± 0.027	0.009–2.891
Poultry and other			
Chicken	402	0.471 ± 0.036	0.009–6.7161
Goose	38	0.747 ± 0.133	0.036–4.208
Duck	30	1.388 ± 0.445	0.071–5.249
Pigeon	36	0.729 ± 0.116	0.018–2.491
Kidney			
Pig	910	2.796 ± 0.107	0.107–51.303
Cattle	285	3.977 ± 0.258	0.383–22.694
Sheep	61	0.507 ± 0.071	0.000–2.028
Muscle			
Mammals			
Pig	998	0.418 ± 0.027	0.000–8.175
Cattle	288	0.436 ± 0.054	0.009–10.586
Sheep	60	0.258 ± 0.035	0.009–1.209
Rabbit	159	0.222 ± 0.035	0.009–1.744
Poultry and other			
Chicken	523	0.427 ± 0.107	0.009–9.136
Goose	62	0.320 ± 0.036	0.009–1.468
Duck	44	0.418 ± 0.062	0.009–1.779
Turkey	55	0.285 ± 0.027	0.009–1.655
Other			
Salami	278	0.489 ± 0.027	0.009–2.438
Sausage	87	0.427 ± 0.036	0.009–3.461
Ham	219	0.222 ± 0.044	0.009–1.317
Canned beef	96	0.898 ± 0.311	0.009–23.966

The amount of absorbed Cd also depends on the site of deposition of Cd present in the polluted air. The majority of Cd deposited in the nasal cavity and larynx will be either removed from the organism with the phlegm coughed up and with the nasal secretion, or will be swallowed by the animal. The ciliated epithelium lining the trachea also helps remove the deposited Cd. A substantial part of the Cd entering the alveoli will be absorbed and get into the blood or lymph (Oberdörster et al., 1980). Humans may inhale substantial amounts of Cd with the cigarette smoke (with 20 cigarettes daily, depending on whether they are filter-tipped or not, 20–30 μg Cd gets into the organism). About half of that quantity (10–15 μg Cd/day) will remain in the lungs. Consequently, the lungs of an active smoker contain much more Cd than those of a non-smoker (Cole and Ilzro, 1980). It has also been observed (Bache et al., 1991) that from the heavy metal containing substances used by potters and dental technicians more or less Cd absorbs into, and accumulates in their organism. The average Cd content of hair samples from 70 potters and 75 dental technicians was found to be 0.28 ppm and 0.17 ppm, respectively. The extreme values varied widely with the working conditions and the use of protective equipment.

Inhaled Cd is especially harmful and toxic to the young organism where it sooner reaches a toxic concentration in the kidney. It is a real cumulative poison: entering the organism through the digestive tract and with its particles of less than 2 μm in size through the lungs, it reaches the highest concentration in the liver and kidneys (Friberg et al., 1979*b*; Kreuzer, 1983; Hecht, 1983; Kleiminger, 1983). The accumulation of Cd is affected by the individual's renal diseases, disturbances of mineral metabolism, and stress conditions (Hapke, 1975; Schinner, 1981).

2.2. Metabolism of Cd in the organism

Cadmium metabolism is difficult to study, as the elimination of that element is slow and its levels are hard to measure in most organs (Fox, 1982). Its absorption and accumulation within the organism of mammals and birds have been studied by many researchers (Pribble, 1973; Weigel et al., 1987; Gruden and Munic, 1987; Tu, 1989; Stolley, 1989; Anke et al., 1977, 1981). The accumulation of Cd and the interaction between Cd and Se accumulation have been studied also under laboratory conditions, in fishes kept in aquaria (Nielsen and Bjerregaard, 1991).

Cd absorbing from the gastrointestinal tract was found to reach its very low maximum concentration in the blood in 2–8 hours (Van Bruwaene and Kirchman, 1979). A substantial part (70–85%) of Cd absorbed into the blood is bound to plasma proteins (mostly to albumin and to a lesser extent to amino acids and polypeptides) and can be detected in the blood elements. Upon acute Cd load the Cd content of the blood plasma and the blood elements may transiently increase

even by an order of magnitude (Hogan and Jackson, 1986). In young red blood cells, Cd binds partly to the heat-stable metallothionein (molecular weight: approx. 6,600), a compound containing many amino acids and readily forming complexes with Cd, partly to haemoglobin, and it accelerates the aging process of erythrocytes (Tanaka et al., 1986). Small amounts of Cd can be detected also in the platelets. In the form of Cd-metallothionein, the blood transports Cd first into the liver and then gradually into other organs, first of all into the kidney where most of it forms a stable complex with proteins at a pH value of 7–10 (Yayawickreme and Chatt, 1990). Other organs contain less Cd (Anke et al., 1981). Cd reaches also the muscles. No major differences have been demonstrated in the Cd content of different muscles (Hecht, 1979). As the placental barrier retains Cd and because the metallothionein contained in the maternal liver and kidney binds the Cd present in the blood, reducing its blood concentration, only little Cd gets into the organs of the fetus (Kelman and Walter, 1977). The Cd content of certain organs of neonates have been shown to vary with the mother's Cd load (Anke et al., 1977, 1984; Neathery et al., 1974; Schramel et al., 1988). Clare et al. (1978) measured the following average Cd concentrations in different organs of newborn fetuses (n = 12–33): liver: 0.07, kidney: 0.08, brain: 0.11, myocardium: 0.07, lungs: 0.12, skeletal muscles: 0.03, bone: 0.08 μg/g dry matter.

In the testes, Cd is bound to a protein containing aromatic amino acids and of low cysteine content, which is functionally similar to the metallothionein of the liver and kidney (Furuta, 1977). As compared to other organs, relatively little Cd is deposited in the bony substance (Gunnarsson, 1983). Sweat and milk also contain Cd in lower concentrations (Fox, 1979).

More than 50% of the Cd is stored in the liver and kidney. The type of Cd storage is different in these two organs. Namely, while the Cd concentration of the liver as transit organ varies with the uptake, the renal cortex accumulates Cd continuously. Upon severe Cd load, large amounts of Cd can be detected in the entire kidney (Hapke et al., 1977). The biological half-life of Cd is 116–206 days, depending on the dose and the species (Moore et al., 1973). In man the half-life may be as long as 15–30 years (Friberg et al., 1975).

The lungs, the pancreas, the spleen and most endocrine glands accumulate smaller amounts of Cd. Relatively little Cd can be found in the brain, myocardium, hair follicles, bone, muscle and adipose tissue (Sumino et al., 1975; Köfer and Fuchs, 1993; Kreuzer and Rosopulo, 1981). In Finland, the average Cd concentration of cattle and pig liver was found to be 66 and 21 μg/kg, respectively (Tahvonen and Kumpulainen, 1993), which were considered low values.

The weekly Cd uptake still tolerable by the adult human and animal organism is approx. 500 μg (Glauser et al., 1976; Vaessen and Fellen, 1986).

In Hungary, the amount of accumulated Cd detectable in different organs of animals has been studied in detail by Régius-Mőcsényi (1991) in order to determine the normal concentrations and draw conclusions to the presence of a Cd load, if any. The most important findings of her investigations are as follow: (*i*) In cows, the kidney contains the largest amount of Cd (2,970 μg/kg dry matter). The liver contains about 25% (739 μg/kg dry matter) while the hairs approx. 1.2% (36 μg/kg dry matter) of the quantity present in the kidney. A significant correlation can be demonstrated between the Cd content of the hair and that of the parenchymal organs: therefore, hair analysis is a suitable indicator of Cd load. (*ii*) There was hardly any individual variation between cows in the Cd content of the haircoat. (*iii*) The organs of sheep contained 0–37% more Cd than those of cattle: the highest concentration was found in the kidney, followed by the liver and the haircoat. (*iv*) The Cd content of the equine kidney (110,000 μg/kg dry matter on the average) was multiple of that measured in cattle and sheep. This was attributed first of all to the long life span of the horse. The horse liver also had relatively high Cd concentration (average: 13,600 μg/kg dry matter), which is attributable to species characteristics among other factors.

The Cd content of major foods of animal origin is shown in Table 10. In Hungary, the Cd content of animal tissues and animal foods was analyzed by Tu (1989) and Sas and Tu (1989) by assaying organ samples collected from farm animals during a period of five years. The results obtained were favourable from the food hygienic point of view. Namely, the highest Cd content was measured in muscle, liver and kidney samples of cattle (0.363, 0.978, 4.9 μmol/kg, respectively). They found strikingly high Cd concentration in the liver of ducks (1.388 μmol/kg). Recently, German authors (Schwarz et al., 1991) have found Cd contamination exceeding the food hygienic upper limit in the liver and kidney of ruminants.

2.3. The elimination of Cd

A substantial part (90–95%) of absorbed Cd gets into the intestine primarily with the bile (Doyle et al., 1972) and through the intestinal villi (Berlin and Ullberg, 1963), and is excreted via the faeces. Upon heavy Cd load, excretion via the urine increases, while it decreases with age (Nordberg, 1975; Friberg et al., 1985; Andresen, 1990). Renal injury reduces the rate of elimination which shows marked individual variation (Kühl, 1976). Excretion of Cd with the saliva and through the skin is limited. Little Cd leaves the organism with the milk and with detached hairs. Bovine milk contains only little Cd (0–5 μg/l) while the Cd content of the colostrum is somewhat higher (Groppel, 1969). The Cd content of milk varies with individual and with the number of lactations. In an experiment with labelled Cd, approx. 2% of orally or parenterally administered Cd was excreted in

the milk in the first five days (Van Bruwaene et al., 1982). The administered Cd was found to bind mostly to the protein fraction of the milk (Van Bruwaene and Kirchman, 1979). According to other authors (Kronemann et al., 1982), the Cd content of bovine pigmented hairs is 0.025–0.05 mg/kg.

3. The toxic effects of cadmium

3.1. General toxic effect of cadmium on different tissues and physiological functions

The toxicity of Cd has been amply demonstrated. This element induces acute and chronic pathological processes depending on the mode and duration of uptake and first of all on the quantity taken up. In monogastric animals, Cd ingested with the feed and drinking water at a dose of 1 mg/kg causes mild while 5 mg/kg Cd gives rise to rather severe clinical signs (Mineral Tolerance of Domestic Animals, 1980). Chronic Cd poisoning is the best studied and has the greatest importance: it occurs upon prolonged uptake of small amounts of Cd. Acute Cd poisoning involves a systemic effect of Cd, while chronic poisoning is characterized by specific effects of Cd exerted on different organs (Andresen, 1990).

In humans, the oral ingestion of a single large dose of Cd gives rise to severe, acute clinical signs (dizziness, vomiting, colicky spasms, diarrhoea and shock) developing in a few minutes (Friberg et al., 1974). The oral administration of a single large dose of Cd has been reported to cause abortion, changes in the reproductive organs, nervous system and other organs. The sensory ganglia suffered damage and became necrotic. Necrosis developed in the atrophic testicle and fetal membranes, while the ovaries became atrophic. In animals that died upon the intraperitoneal administration of 0.5–1.0 mg Cd salt per kg body mass, the most severe lesions (necrosis, degeneration) were found in the liver.

Accumulation of Cd in the organs upon prolonged administration of small amounts depends on the amount taken up (Schenkel, 1990), as well as on the sex and health status of the animal. Male goats incorporated more Cd into their body than did female goats under identical conditions. This finding was explained by the antagonistic effect exerted by oestrogen hormones on Cd incorporation (Anke, 1986; Anke et al., 1982). In their investigations using a Cd isotope, Miller et al. (1968) could not confirm any effect of sex on Cd accumulation. Jopek et al. (1980) reported that the liver and lymph nodes of leukaemic cattle had elevated Cd levels.

Cd sensitivity varies with species. While in monogastric animals 30 mg Cd per kg of feed will produce pathological changes in the organs, in poultry 50 mg Cd per kg of feed is required for that (Hapke, 1992). For instance the rat is more

tolerant to Cd and can endure a higher dose of it without developing the signs of gastroenteritis. This is explained by differences in Cd absorption; however, it is also related to the composition of the diet fed and the animal's state of nourishment (Fox, 1974; Miller et al., 1968; Schenkel, 1986). In rats, Cd poisoning may take a protracted course, giving rise to an injury of the liver and other organs without causing changes in the intestinal tract. At the same time, in humans the gastrointestinal signs are dominant. In humans, vomitus may develop already after the ingestion of drinking water containing 15 mg Cd per litre.

The pathological effect exerted by Cd on the testis was first reported in the 1920's (Alsberg and Schwarze, 1919–1920). After that, almost four decades had to pass until it was demonstrated in animal experiments that the parenteral administration of small amounts of $CdCl_2$ caused severe changes (degeneration, haemorrhagic necrosis) in the testicles of male rats and in the fetal membranes of pregnant female rats (Parizek, 1983; Parizek and Zahor, 1956).

Prolonged oral intake of low doses of Cd may damage the villi of intestinal epithelial cells playing an important role in absorption (Mason et al., 1977), and may cause renal and heart injury (Schroeder et al., 1965). Other authors have observed pulmonary fibrosis and myocardial changes (Petering et al., 1979). Cd exerts an effect on the activity of various enzymes, binds to certain amino acids, and impairs protein synthesis and cell division (Sandstead et al., 1983). All these effects indicate that Cd exerts deleterious effects already at low doses.

The biochemical mechanism of these adverse effects is hardly known yet (Mertz, 1986). According to certain data, the prolonged uptake of low Cd doses inhibits the normal functioning of metallothionein; thus, Cd accumulates and causes renal injury which, in turn, leads to prolonged proteinuria accompanied by amino-aciduria, glycosuria and phosphaturia. The excretion of beta-2-globulin occurring during proteinuria in itself does not cause severe health injury; however, it indicates that the kidney is saturated with Cd and that any further Cd uptake may lead to a severe impairment of renal function.

In Japanese women, the renal function, the consumption of Cd-containing foods and protein deficiency cause symptoms of osteomalacia and osteoporosis (itai-itai; Underwood, 1977). Unbalanced vitamin D and iron supply may also contribute to the development of the above-mentioned bone lesions (Roels and Lauwerys, 1983). In Sweden, men affected with Cd-induced renal injury developed nephrolithiasis at a high Ca intake (Piscator, 1982).

In animals, a short-term inhalation of Cd gives rise to lung injury, in severe cases to pulmonary oedema of fatal outcome. The inhalation of air containing 5 $\mu g/m^3$ Cd for 8 h proved to be fatal (Holden, 1980).

Primarily in highly developed industrial countries the prolonged uptake of Cd usually leads to renal injury and proteinuria, irrespective of whether Cd is taken up orally or by inhalation. After the prolonged inhalation of low doses of Cd the renal lesions are dominant. The uptake of large doses of Cd by animals with

the feed results in diverse symptoms including growth disturbances, anaemia, poor bone calcification, severe renal and testicular injury, cardiac dilatation, hypertension, and fetal injury. These adverse effects have been reported to occur in almost all species of animals exposed to a Cd load of varying severity, including cattle (Johnson et al., 1977; Wright et al, 1977; Powell et al., 1964), sheep (Mills and Delgarno, 1972; Doyle and Pfander, 1975), goat (Anke et al., 1970), pig (Freeland and Cousins, 1973; Hansen and Hinesly, 1979), poultry (Freeland and Cousins, 1973; Learch et al., 1979; Pritzl et al., 1974; Sharma et al., 1979), Japanese quail (Richardson and Fox, 1974; Mason et al., 1977; Jacobs et al., 1978), dog (Fairchild et al., 1977), rat (Perry et al., 1977) and mouse (Schroeder and Mitchener, 1971).

3.2. Specific effects of Cd on different organs

In the *liver*, which accumulates nearly half of the Cd absorbed during a Cd load, sooner or later tissue changes associated with functional disorders will develop. By gross pathological examination, it first develops degenerative lesions followed by fibrosis, if there is sufficient time for the latter to develop. Histopathological examination reveals focal necrosis, karyopycnosis, vacuolation of the Kupffer's cells, infiltration by inflammatory cells around the bile sinuses with deposition of fibrin and debris, and structural disintegration of the endoplasmic reticula (Failla et al., 1979). The limiting membranes (cell membranes) sustain injury; as a result, the activity of numerous enzymes (gamma-glutamyltranspeptidase, aspartate aminotransferase and sorbitol dehydrogenase) increases in the plasma (Whanger, 1973). Several authors (Hecht, 1986; Friel et al., 1987) reported that the parenteral administration of Zn solution before Cd exposure markedly diminishes the toxic effect of Cd administered intravenously at a lethal dose. While the liver of animals not receiving Zn pretreatment could detoxify approx. 60% of the injected Cd, that of animals pretreated with Zn detoxified 90% of the injected Cd in metallothionein binding (Friel et al., 1987).

When studying the nephrotoxic effect of Cd, we have to set out from the fact that during Cd exposure the concentration of metallothionein-bound Cd multiplies in the *kidney* (Friberg, 1950). Cd accumulates primarily in the renal cortex, in epithelial cells of the proximal tubules. The Cd content of the urine will rise only several days later (Scott et al., 1987), indicating the severity of the renal dysfunction induced (Hoffmann et al., 1979). Besides the size of the Cd load, the severity of renal injury and dysfunction depends on the route and duration of uptake and on the species characteristics. Microscopic renal injury caused by ionized Cd is characterized by vacuolar degeneration of the tubular epithelial cells and the appearance of electron-dense lysosomal formations in them (Felley-Bosco and Diezi, 1987). Similar changes may be produced by Cd-thionein. Renal injury ac-

companied by impaired amino acid and glucose reabsorption is irreversible (Friberg, 1950). Aggravation of the renal injury is indicated by the increased excretion of proteins of low molecular weight (e.g. beta-2-microglobulin), and of certain amino acids (proline) within the proteinuria (Tohyama et al., 1986).

According to the WHO, the critical cadmium concentration of the human renal cortex is 200 μg/g (Kucharz, 1988), while the Cd concentration still tolerable from the food hygienic point of view is 1 mg/kg wet mass.

Among the effects exerted by Cd on the *reproductive organs* and on *development* the haemorrhagic degeneration of the atrophic *testicles* and *ovaries* in acute Cd salt poisoning should be mentioned (Mahaffey et al., 1977). Metallothionein-bound Cd does not produce such effects. This explains why these lesions occur in a milder form in chronic Cd poisoning. Upon prolonged Cd exposure the Cd accumulated in the interstices leads to degeneration of the endothelial lining of blood vessels, impaired blood supply of the organ, and inflammatory infiltration of the interstices. Ultrastructural changes include karyopycnosis, degeneration of the endoplasmic reticula, and increased vacuolation. In young animals these changes occur in more severe form. At the early stage of Cd intoxication breeding bulls show impaired fertilizing capacity, accounted for by the high number of injured and immotile spermatozoa (Anke et al., 1977).

Cd also accumulates in the *placenta* and, especially in late pregnancy, small amounts of Cd cross the placenta and reach the *fetus* (Sonavane et al., 1975). Major accumulation of Cd in the fetal organism is prevented by metallothionein present in the maternal liver and kidney, by keeping the Cd concentration of the blood on a low level for a long time (Kelman and Walter, 1977). Recently the fetotoxic effect of Cd is attributed to an indirect, rather than a direct, action of Cd, namely that Cd competitively inhibits transplacental Zn transport. Reduced maternal feed intake and impaired feed utilization caused by Cd also play a role in the fetotoxic effect. As a result of prolonged Cd uptake, the small blood vessels of the uterine wall become damaged. The uterine wall thickens, the implantation of the ovum and the oxygen and blood supply of the fetus become impaired (Nolan and Shaikh, 1986). Ward (1993) studied the effect of smoking on pregnancy in pregnant women who were active (n = 175) or passive smokers (n = 108) or non-smokers (n = 185). It was found that the newborn babies born to active smokers weighed 279 g less while those of passive smokers 499 g less at birth than those delivered by non-smoking mothers. The average Cd concentration of the placenta was 0.41 μg/g in non-smokers, 0.99 μg/g in active smokers and 1.03 μg/g in passive smokers. At the same time, the Zn content of the placenta underwent a corresponding decrease.

The glandular tissue of the udder allows only small amounts of Cd to be secreted into the milk; thus, the Cd exposure to neonates from that source is negligible (Piscator, 1983). The average Cd content of milk is 0.1 mg/l, 96% of which is bound to protein while 3% to lipid phytase.

Cd causes functional damage in the *muscle tissue* and in the *myocardium*, partly due to the inhibition of myosin phosphorylation, partly resulting from a disturbance of acetylcholine and Ca metabolism (Deudal, 1987; Itokawa et al., 1974). In the myocardium, Cd alters the membrane potential of Purkinje's fibres, thus reducing atrioventricular conductivity. This effect is based on the interaction of Cd with the SH groups of the membrane of impulse-conducting cells and on the resulting inhibition of membrane-bound enzymes (e.g. ATPase). As a result, myocardial ischaemia develops which may lead to myocardial infarction (Sturkie, 1973).

The mechanism of the hypertension observed during Cd load by several authors (Schroeder et al., 1965; Perry and Perry, 1974; Perry et al., 1976; Ohanian et al., 1978) has not been fully elucidated yet.

In some cases, Cd has been reported to cause focal haemorrhages, vacuolation of the endothelial cells of blood vessels, and degeneration of the cerebral ganglia in the *central nervous system.*

The effect exerted by Cd load, Cd excess and simultaneous protein deficiency on the ossification procedures (itai-itai disease) has been thoroughly studied primarily by Japanese authors (Takeuchi, 1973). Several explanations have been proposed for the development of *bone lesions* occurring in the form of osteomalacia and osteoporosis. Reduction of the active metabolite(s) of vitamin D_3 to a minimum due to renal injury, and the slower formation of bone collagen are regarded as the primary effects of Cd. The secondary effects of Cd include insufficient osteoblast formation and disturbed calcification (Lorentzon and Larsson, 1977; Furuta, 1977; Nordberg, 1975). In addition to lesions typical of osteoporosis and osteomalacia, in such cases the mucopolysaccharide content of the epiphyseal cartilage decreases, the cartilage becomes thinner, in the metaphysis the number of bone trabeculae decreases and the red marrow undergoes fatty degeneration. Microscopic examination reveals eosinophilia and an increase in the number of osteoclasts (Ahokas et al., 1980).

3.3. Other effects of Cd exposure

Exposure to Cd may cause damage to the Langerhans' islets of the pancreas and thus suppress *insulin secretion* (Ghafghazi and Mennear, 1975). Indirectly, Cd reduces the activity of cholecystokinin and serum cholesterase and raises the blood glucose level.

Yoshizuka et al. (1991) studied the toxic effects of Cd intraperitoneally administered to pregnant rats for 4–6 days, and found that in Cd-exposed animals the serum T3 and T4 level significantly decreased. On the 5th day of Cd exposure damage of the mitochondria and endoplasmic reticula was observed in the epithelium of the thyroid follicles: large intracellular vacuoles appeared and mitochon-

drial swelling was seen. In pregnant rats, corneal oedema has also been observed as the result of Cd load (Yoshizuka et al., 1990).

The *other effects* of Cd include anaemia, which is explained by the Cd-induced disturbance of Fe absorption (Piscator, 1974). Cook et al. (1975) demonstrated increased sensitivity of Cd-exposed rats to the endotoxins of certain Gram-negative bacteria. An *immunosuppressive effect* of Cd has also been described (Kranjc et al., 1983).

3.4. Carcinogenic, mutagenic and teratogenic effects of Cd

The *carcinogenic effect* of Cd has been suggested on the basis of Cd accumulation observed in some tumorous tissues in the practice (Rudd and Herschman, 1979), and has been confirmed by some experiments (Goering et al., 1985). This effect of Cd has been convincingly demonstrated in rats, which developed lung cancer after inhaling, over a period of 18 months, air which contained 12, 25 or 50 μg $CdCl_2$ per m^3 (Takenaka et al., 1983).

The intratracheal administration of cadmium oxide resulted in the development of mammary tumours in female rats (Sanders and Mahaffey, 1984). The injection of $CdCl_2$ solution into the prostate led to the development of tumorous lesions in the tissues (Scott and Aughey, 1979). Pancreatic tumours could also be produced in a similar manner.

Orally administered Cd is at least 200 times less carcinogenic than Cd getting into the organism by inhalation.

Under field conditions, a carcinogenic effect of Cd has been most commonly implicated in the aetiology of lung and prostatic cancer, in places where people are exposed to environmental pollution with Cd (Friberg et al., 1979*b*).

In experiments conducted *in vitro* and *in vivo*, Cd and its organic complexes enhanced *heterogeneous cell mutations* and the emergence of abnormal cell lines, and increased the incidence of chromosomal aberrations (Fontana et al., 1987). Cd ion drives out Zn and Cu from the enzyme superoxide dismutase, thus inhibits the activity of that enzyme, and damages the DNA by increasing the free radical concentration of the tissues. Cd ion exerts a similar effect also by inhibiting the Se-dependent glutathione peroxidase enzyme (Wong, 1988).

Cd has *teratogenic effects* and acts as an antagonist of Fe, Zn and Ca. It is also fetotoxic: as its presence is accompanied by poor Zn supply status, Cd may result in stillbirths and certain congenital abnormities.

In laboratory animals exposed to a Cd load the following teratogenic effects have been observed: abnormities of the fetal membranes and placenta, ossification disorders (distorted bones), anephria, urinary bladder atrophy, malformations of the genital organs and heart (Holt and Webb, 1987).

3.5. Effect of Cd on the immune status

Cd introduced into the organism either orally or by the respiratory route inhibits the enzymes and the antigen-recognizing ability of cells participating in the cell-mediated defence mechanisms; thus, it exerts an *immunosuppressive effect.* According to the current state of knowledge (Koller et al., 1975; Krzystyniak et al., 1987) it exerts this effect by the following pathomechanisms: it damages the cells of both the bone marrow and the circulation, reduces the ratio of rosette-forming cells and plasmocytes, alters the number and phagocytosing ability of circulating lymphocytes and other leucocytes, reduces the number of lymphocytes and the rate of DNA synthesis in the cortex of the thymus, and the Cd ion damages humoral factors facilitating IgG synthesis and the activity of RES cells.

3.6. Interaction of Cd with different mineral elements and vitamins

The composition as well as the protein, vitamin and mineral element content of diet exerts a major influence on the absorption and effect of toxic heavy metals (Schenkel, 1983; Kollmer and Berg, 1988).

In experimental animals, *selenium (Se)* administered before or simultaneously with Cd was found to prevent the deleterious effect of Cd on the testicles and placenta (Kar et al., 1960; Parizek et al., 1968). Se has been found to exert a beneficial effect on Cd-induced chromosomal aberration (Mukherjee, 1988). Due to its complex formation with metallothionein (Bruckner, 1988), Se reduces the retention of $CdCl_2$ in different organs, as it inhibits the protein complex formation of other metals including Cd. In acute or chronic Cd toxicosis, Se compounds diminish the orchiotoxic and enzyme-inhibiting effects of Cd (Ohta et al., 1993) through the formation of a Cd-Se-protein complex.

Zinc (Zn) is an element which occurs together with Cd in nature. Zn deficiency of the organism facilitates the accumulation of Cd in the liver (Fox et al., 1984). At the same time, the secondary deficiency of Zn may give rise to lesions of the skin and haircoat and a depletion of Zn from the bones.

In broiler chickens fed a diet supplemented with 50 mg Zn per kg not only the absorption of Cd decreased, but 40% less Cd accumulated in the liver and kidneys than in control chickens (Bundscherer, 1984). The protective effect of Zn against the deleterious effects of Cd ingested with the diet has been confirmed by Jacobs et al. (1983).

In quails, Cd administered in capsule formulation and Zn administered at increasing doses resulted in an expressed reduction of Cd accumulation in the kidney and in numerous other organs including the stomach, small intestine, and liver (Fox et al., 1979).

Using radiolabelled Cd (^{109}Cd), Jacobs et al. (1974*a, b*) found that when feeding to quails a diet supplemented with 30 mg Zn, 12 mg Mn and 4 mg Cu, these elements reached much lower concentrations in the liver and kidneys if the diet contained 0.02–1.02 mg Cd.

According to Runkel and Payer (1984), the accumulation of Cd in the liver is influenced also by the chemical form of that element, in so far as Zn has less influence on the accumulation of Cd bound in an inorganic complex. The protective effect of Zn depends on the amount of Cd administered, and accumulation is correlated with the amount of Cd ingested: when feeding a Zn-deficient diet, the uptake of a low Cd dose led to the signs of Zn deficiency; at the same time, these signs did not develop if the feed contained adequate amounts of Zn.

Studying the interaction of Cd with other microelements, Kozlowska et al. (1993) found that in Cd exposed rats showing poor development the concentration of *iron (Fe)* in the bones did not change while in the liver and duodenum it was significantly lower than in the control animals. Similarly, in rats exposed to a heavy Cd load the Fe concentration of the kidneys also markedly decreased. The Zn concentration of the bones did not decrease while that of the liver and kidneys increased.

The accumulation of Cd in the organism may cause the development of secondary *copper (Cu)* deficiency (Groppel et al., 1979), resulting in the birth of non-viable offspring and possibly abortion. In such cases the Cu concentration of all organs is lower.

Large doses of Cd reduce the absorption of Cu from the intestine (Davies and Campbell, 1977). Cd has been observed to exert the same effect on the absorption of Zn. Namely, in animal experiments it was found that after the uptake of Cd in doses as low as 1.5 mg/kg the ceruloplasmin content of the plasma and the Cu content of the kidney significantly decreased. That effect became more expressed when the dose of Cd was raised. Fox et al. (1984) observed that in broiler chickens with mild Cu deficiency the retention of Cd increased in the kidney but did not change in the liver.

No appreciable Cd accumulation was seen in the liver and kidney of broiler chickens if the dietary intake of *manganese (Mn)* was twice the requirement; however, Cd concentration increased in the jejunum and ileum (Jacobs et al., 1983). Other authors (The Task Group on Metal Interaction, 1978) reported enhanced Cd absorption from the intestine if the Mn content of the diet was increased. On the other hand, after administration of 0.02 mg or more Cd in the diet a significant reduction occurred in the Mn concentration of organs (Gruden and Munic, 1987).

The simultaneous administration of high doses of Mn and Cd via the drinking water reduced the Cd concentration of the liver and kidney. This did not occur if only Cd was administered (Sarhan et al., 1986). Elsenhans et al. (1987) ob-

served an interaction between Cd and Mn only in the intestine but found a Cd–Fe interaction in almost all organs.

The possible interaction between *vitamins* and Cd retention was studied in poultry. Our knowledge of the effects exerted by vitamins on Cd absorption is scanty. Vitamin E and vitamin B_6 administered in the diet at different concentrations did not exert any effect on the amount of Cd retained in the liver and kidney. At the same time, Cd load reduced the vitamin C reserves of the organism.

3.7. Study of the mechanism of toxicity by determination of the activity of some microbial enzymes

The *catalase* enzyme produced by certain microorganisms is known to be capable of converting the toxic hydrogen peroxide (H_2O_2) into water and oxygen (Baird-Parker and Davenport, 1965). In their experiments, Korkeala and Sankari (1980) used Cd-sensitive and Cd-resistant *Staphylococcus aureus* cultures to study the effect of catalase activity on Cd toxicity. They found that in cell cultures the catalase activity (production) of Cd-resistant *S. aureus* was higher than that of the Cd-sensitive strain. At the same time, Cd did not affect the catalase activity of bovine liver or the catalase production of other bacterial cells. Catalase was found to diminish the toxicity of Cd, especially in the case of plasmid-negative, Cd-sensitive *S. aureus*. Catalase produced in large amounts by Cd-resistant *S. aureus* may be one of the factors involved in the enhanced resistance of cells to the toxic effect of Cd. The results suggest that Cd, together with H_2O_2, causes oxidative damage to the cells if catalase is not present in a quantity sufficient for converting all the H_2O_2 present.

Korkeala et al. (1981) studied the effect of *cystine* and *sodium selenite* on Cd toxicity in *S. aureus* cultures under laboratory conditions. That study was prompted by the observation of Tynecyca et al. (1975) and Gauthier and Flatau (1977) that the Cd uptake of staphylococci pretreated with cystine decreased. In another experiment, the same authors (Korkeala et al., 1981) found that although cystine decreased the bactericidal effect of Cd, it had no effect on the capacity of *S. aureus* to take up Cd. In a liquid medium, sodium selenite solution also did not reduce the Cd uptake of *S. aureus* bacteria and, contrary to the observations of other authors, nor did it diminish the toxic effect of Cd.

Korkeala (1980) studied the effect of vitamin E on Cd toxicity in *S. aureus* cultures. The addition of vitamin E to Cd-containing liquid cultures of *S. aureus* shortened the growth period of *S. aureus*. In that case vitamin E obviously acted as an antioxidant against the oxidative damage caused by Cd and by H_2O_2 produced by the bacteria.

Noever et al. (1992) studied the toxic effect of Cd and the effects of the Cd-antagonistic elements Ni and Cu in cultures of *Tetrahymena pyriformis* as a biological indicator.

Studying the effects of Cd in cell cultures, Szymanska and Laskowska-Klita (1993) observed that the addition of 0.5 mM Cd to the cultures did not affect the activity of lipid peroxidase and glutathione-dependent enzymes, while at a dose of 1.5 mM it enhanced the activity of both lipid peroxidase and lipid reductase. The activity of the other glutathione-dependent enzymes did not change even in the latter case.

4. Study of the effect of Cd in some animal experiments

4.1. Studies on poultry species

Birds are characterized by low sensitivity to Cd load. In an experiment with 274 day-old broiler cockerel chicks, Stolley (1989) studied the effect of some vitamins (E and B_6) and microelements (Cu, Se, Mn, Zn) on the accumulation of Cd in the kidney. The vitamins and microelements were applied at varying concentrations and in some cases in combination. Cd was administered in the form of $CdCl_2$ in the diet which contained a total of 3 ppm Cd. Zn, Cu and Mn were given as acetate salts while Se in the form of sodium selenite. The birds' health status, body mass and feed utilization were monitored throughout the 21-day feeding trial. Crystalline vitamin E manufactured by Merck and pyridoxine (vitamin B_6) were homogeneously mixed in the soybean- and maize-based diet of the different groups. At the end of the experiment the birds were sacrificed, necropsied and their organs were used for determining the concentration of the above vitamins and microelements.

The main results of the experiment were as follow: (*i*) The 3 mg Cd per kg of feed dose was comparable to that generally taken up by humans and animals in chronic Cd poisoning. (*ii*) Vitamin E and pyridoxine administered at different concentrations did not affect Cd retention. (*iii*) A single 5 ppm dose of Se reduced Cd retention in the kidney. (*iv*) Changing the Mn concentration of the diet had no spectacular influence on the Cd content of the kidney. (*v*) Elevated dietary Cu content (240 ppm) resulted in significant (75–240%) Cd accumulation in the kidney. (*vi*) The Cd content of the kidney was found to be 616–2,718 mg/kg dry matter.

Pritzl et al. (1974) studied the effects of a Cd load on 196 two-week-old Leghorn cockerels. In the first experiment, 100 chickens were divided into five groups fed different levels (0, 400, 600, 800 and 1,000 ppm) of dietary Cd in the form of $CdCO_3$. In the second experiment, 96 cockerels were assigned to four groups. Two groups served as control fed a diet without Cd supplementation,

while the diet of the remaining two groups was supplemented with 700 ppm Cd. The main findings were as follow: (*i*) In the birds fed 700 ppm Cd, the volume of the gastrointestinal tract and the mass of the kidney and of the gizzard lining increased. (*ii*) In the birds fed Cd, alterations were observed in the colour of organs and in the tonicity, Cd concentration and Zn content of the muscles. (*iii*) Parallel to the increasing Cd load, the body mass gain of 14- to 34-day-old chickens decreased and their feed utilization was lowered. (*iv*) In the commercial diets, Cd doses exceeding 400 ppm proved to be highly toxic. (*v*) The LD_{50} of Cd in 2-week-old chickens was found to be 565 ppm.

Bruckner (1988) studied the effect of sodium phytate and Se on the metabolism and renal accumulation of Cd in broiler chickens. As regards the accumulation of Cd in the kidney, he obtained results consistent with those reported by other authors.

Sharma et al. (1979) fed a diet containing 0.3, 1.9 and 13.1 ppm Cd to Leghorn layers for a period of 6 months. In laying hens exposed to such a Cd load, Cd accumulation occurred neither in the egg nor in the bones. At a slightly higher Cd load, Cd accumulation was demonstrated in the muscles in the 6th month of the trial. The highest concentration of Cd was measured in the liver and kidney; the Cd content of these organs was closely correlated with the dose and the duration of exposure.

Learch et al. (1979) studied the effects of Cd exposure in broiler chickens and laying hens fed a diet of varying Cd concentration (0.3, 12 and 48 μg/g) for a period of 6–12 and 48 weeks. The most important results of that experiment were as follow: (*i*) the diet containing 48 μg/g Cd was found to be slightly toxic. (*ii*) All the three levels of Cd load increased the Cd concentration of the kidney, while the Cd content of the liver and muscles was raised by the two higher doses only. (*iii*) The Cd content of eggs was found to be very low. Measurable increases in egg Cd content were found only in the eggs laid by hens fed a diet containing 48 μg/g Cd in dry matter. (*iv*) As a result of the Cd load, egg production and eggshell thickness decreased.

It is known that if the diet of layers contains large amounts of heavy metals, these elements will pass into the eggs (Stadelman and Prati, 1989). Holcman and Smodis (1993) studied the passage of four microelements (As, Cd, Hg and Pb) into the eggs of laying hens kept under natural conditions or in closed housing. They found that in the lyophilized samples As, Cd and Hg accumulated in the egg-white while Pb in the egg-yolk. Of the four elements studied, Cd, As and Pb was present in the fresh egg-yolk in higher concentrations than in the fresh egg-white. This did not hold true for Hg. The eggs laid by hens kept under natural conditions in an environment exposed to Cd pollution contained higher amounts of the above four heavy metals than did those laid by hens fed commercial diets and kept in closed housing.

4.2. Studies on ruminants

Smith et al. (1991*a*) studied the metabolism of Cd and its effects on the organism of newborn calves in a trial conducted with 36 Holstein-Friesian primiparous heifers. The heifers were divided into three groups and fed a diet of identical composition, with the difference that the diet of the control group was not supplemented with Cd (its Cd content was 0.25 ppm), while the diet of the two experimental groups was supplemented with $CdCl_2$ to give Cd levels of 1 ppm (low Cd load) and 5 ppm (high Cd load) per kg dry matter.

In all three groups, biopsy samples were taken from the liver, renal cortex and abdominal muscles of half of the animals 10 days before the feeding trial, within 5 days after calving and 394 days thereafter, and were assayed for four elements (Cd, Zn, Fe and Cu). Blood, liver and muscle samples were taken from the heifers within five hours after calving and from the calves of all heifers before they sucked colostrum. The most important results obtained were as follow: (*i*) As a result of the 5 ppm Cd load, the Cd content of the kidney increased 62-fold (from 2.61 to 160.63 μg/g dry matter), that of the liver 27-fold (from 0.46 to 12.58 μg/g dry matter), while that of the muscles 4-fold (from 0.06 to 0.23 μg/g dry matter). (*ii*) Zn content of the kidney increased while its Fe content decreased. Cu content of the liver decreased in both experimental groups. (*iii*) In the liver of calves born to heifers exposed to 5 ppm Cd, the concentration of Cd hardly changed while that of Cu and Zn considerably decreased (by 29–43%). In calves of that group, packed cell volume (PCV), haemoglobin concentration and the Cu level of the serum decreased, while serum Zn concentration markedly increased (by 55%). Blood urea nitrogen also increased (by 63%). In summary: exposure of the heifers to 5 ppm Cd during pregnancy did not alter the Cd concentration of the organs of newborn calves but reduced the Zn and Cu content of their liver. (*iv*) No teratogenic effect of Cd was observed in that experiment.

Other findings obtained by Smith et al. (1991*b*) in their Cd exposure experiment performed with the above-mentioned primiparous heifers included the following: (*i*) The uptake of 1 or 5 mg Cd per kg feed did not affect the Cd concentration of the colostrum and of milk samples collected on day 150 of lactation. The Fe, Cu and Zn concentration of the colostrum and milk did not change either. (*ii*) The pH as well as the Zn and K content of the urine were lower in animals fed a diet containing 5 ppm Cd. (*iii*) The Cd accumulation observed in the kidney and liver on day 394 did not increase further by day 554. At the same time, Cd accumulation (a concentration increase from 0.33 to 2.42 μg/g dry matter) was detectable in the adrenal gland, spleen, ovary and mainly in the uterus and pancreas of animals exposed to a high Cd load. (*iv*) Prolonged Cd exposure of the cows exerted no influence on the Cd content of products serving for human food.

Kosla et al. (1993) studied the correlation of Cd with some other elements in serum samples from dairy cows. An important finding of that study was that at

a higher Zn concentration the Cd content of the serum dropped approximately to half. This supports the observation that high dietary Zn content and the consequent higher absorption of Zn diminishes the adverse effects of Cd. The blood values also indicated that Cd had no major effect on the Ca, inorganic P, Mg and Cu content of the serum.

Using radiolabelled Cd, Miller et al. (1968, 1969) studied the metabolism of Cd in young goats. The Cd concentration of the tissues was determined on day 14 after a single intravenous Cd dose (given as $CdCl_2$). It was found that (*i*) 0.3–0.4% of the administered Cd was retained in the organism; half of that amount was retained in the liver, one-fourth in the kidney, while the majority in the intestinal tissues and content. (*ii*) Only low amounts of Cd were concentrated in the bones, muscles, blood and skin. (*iii*) High quantities of Cd taken up with the feed containing 100 ppm Cd significantly decreased the Cd content of tissues of the digestive tract, but no concentration changes were detectable in other organs. (*iv*) The amount of Cd excreted in the faeces decreased in a linear manner; an especially rapid reduction occurred in the 2nd week. (*v*) On day 21 of Cd exposure the Cd content of the kidney, duodenum and liver was 62, 28 and 18 ppm (expressed for dry matter). The corresponding values of the control animals were 2.7, 2.5 and 1.6 ppm. (*vi*) In ruminants, the mechanism of Cd absorption and excretion is not fully understood; it is known, however, that absorption is very low.

Studying the Cd metabolism of domestic animals, Schenkel (1988) published a detailed review of the literature on Cd metabolism in the bovine. That work contains a detailed discussion of Cd metabolism (absorption, elimination, retention, and distribution within the organism) and described the accumulation of Cd in some organs. It supplies data to demonstrate the possibility and degree of "Cd contamination" of some animal products representing important food sources, among them of milk. Finally, on the basis of experimental results, it lists the endogenous and exogenous factors which may affect Cd accumulation in the organism.

Using radiolabelled Cd, Schenkel (1990) himself concluded metabolic studies in small ruminants (sheep and goats). The feeding trials of different duration and conducted with feeds of different composition and supplemented with different doses of Cd yielded the following general statements: (*i*) The rate of Cd absorption was lower than 5%. (*ii*) Because of the different Cd compounds used, the varying Cd concentration of feeds derived from areas subjected to different types of fertilization, the differences in the intensity of feeding and the duration of the trials, and the deviation of the results of analyses, it was impossible to perform a realistic comparison of Cd amounts retained in the different organs, especially at the lower Cd level applied. (*iii*) Among the exogenous and endogenous factors affecting Cd retention, the author stresses the importance of certain feed additives and mineral as well as trace element supplements.

4.3. Studies on rabbits

Based upon the Zn and Cd concentrations measured in the blood and four different organs of growing rabbits in experiments using radiolabelled Zn and Cd, Cotzias et al. (1961) confirmed that Zn acts as an antimetabolite of Cd.

Borgman and Lightsey (1982) conducted a trial with 4 × 10 adult rabbits of mixed sex, and studied the effect of hard drinking water and the Cd dissolved in it (5 mg/l) on lipid metabolism and cholelithiasis. During the 12-week experiment, rabbits of one group were given drinking water which contained 500 mg Ca and 100 mg Mg per litre. The drinking water given to rabbits of the other group was supplemented with 5 mg Cd per litre. In the first case the control group received distilled water while in the second it was given distilled water containing 5 mg Cd. The following results were obtained: (*i*) In rabbits receiving hard water the Ca content of the haircoat increased while its Cd content did not rise, not even in rabbits receiving Cd-supplemented drinking water. (*ii*) The consumption of Cd-containing drinking water increased the Cd concentration of the liver and kidney, which was not affected by the hardness of the water. (*iii*) As a result of Cd exposure, the cholesterol concentration of the liver and the severity of cholelithiasis decreased. (*iv*) The results suggest that the consumption of hard drinking water cannot reduce the incidence of cardiovascular diseases by allowing less Cd to be absorbed from the drinking water.

In organs (kidney, liver, muscle, hair) of brown hares from different regions of Germany Lindner (1989) studied the Cd content and the endogenous as well as exogenous effects that might influence the Cd concentrations. The main findings were the following. (*i*) The Cd content of the hair was relatively low even in animals living in a heavily Cd polluted area. (*ii*) The average Cd concentrations of the different organs, calculated for crude mass, were as follow: kidney 0.445 mg/kg, liver 0.054 mg/kg, diaphragm 0.003 mg/kg, abdominal muscle 0.006 mg/kg. (*iii*) Cd concentration was affected by endogenous factors including the age, sex, body mass and health status of the animals and by exogenous factors such as the season of the year. For example, Cd accumulation took place continuously in the kidney and liver, but gradually from October to December in the hair. (*iv*) The geological conditions (e.g. industrial region) exerted a major influence on Cd accumulation. (*v*) In industrial regions mainly the Cd content of the kidneys was higher. (*vi*) As the regions drawn under study hardly differed in geographic, economic and climatic conditions, the effects of these conditions, if any, could not be substantiated.

Tu (1989) carried out Cd exposure experiments on 2-month-old male rabbits of 2,238 g average body mass. Five groups, each containing 4 animals, were formed. The untreated (control) group received a normal rabbit diet (Cd content 0.011 mg/kg). Rabbits of experimental group 1 were administered daily doses of 3 or 6 μmol Cd per kg body mass for a week. To rabbits of group 2 Cd was admin-

istered by dropping it onto the diet, while in group 3 daily doses of 36 and 180 μmol Cd per kg body mass, respectively, was administered via the drinking water for 2 weeks. Rabbits of group 4 received 36 μmol Cd in the diet plus the same dose of Na_2EDTA. After Cd administration for 7 or 14 days, blood and urine samples and samples from six organs (liver, kidney, thigh muscle, femur, adrenal gland, testicle) were assayed chemically for several mineral elements. The following main findings were obtained: (*i*) The mass and Cd content of the adrenal glands significantly increased in the case of both (subcutaneous and oral) routes of administration. (*ii*) The Cd content of liver, kidney, testicle, muscle and bone samples typically showed a higher increase after subcutaneous Cd administration. (*iii*) As a result of subcutaneous Cd load, the Zn content of the tissues trebled. (*iv*) Parenteral Cd administration was followed by a substantial reduction in the Ca and P content of the bones. The author explained this by enhanced Ca and P excretion due to the Cd load. (*v*) As a result of dietary Cd exposure and the simultaneous administration of Na_2EDTA the urinary excretion of Cd very markedly increased; at the same time, the Cd content of the kidney and adrenal gland was lower in these animals. (*vi*) In animals exposed to Na_2EDTA and Cd, the Cu concentration of the blood plasma markedly decreased while that of the urine remarkably rose. (*vii*) In rabbits exposed to Cd and treated with Na_2EDTA, the Ca concentration of bone-tissue did not differ markedly from that of rabbits receiving Cd alone; however, it was significantly lower than the corresponding value of the untreated control rabbits. (*viii*) As a result of oral Cd load, serum AST and ALT activity rose parallel to the dose of Cd administered, indicating membrane injury and dysfunction of the liver cells. (*ix*) Oral administration of Cd increased the metallothionein content of the liver and kidney by almost one order of magnitude, while the glutathione content significantly decreased in both organs.

Fekete et al. (1994*a*) conducted an experiment involving an alkali clay soil fertilized with heavy metal salts and used for carrot growing. Of the 13 treatments they selected those in which the given microelement appeared in the carrots at an important concentration (Mo 39.00, *Cd 2.30*, Pb 4.01, Hg 30.00, and Se 36.20 mg/kg). Digestion trials were carried out using New Zealand White growing rabbits. The experiment lasted 20 days. At the end of the feeding trial, blood and tissue samples were taken. The nutrient digestibility of the carrot samples was significantly ($p<0.05$) higher than that of the rabbit concentrate. Of the carrots tested, those containing lead showed the lowest protein digestibility (2–3 units lower than the controls). The digestion coefficients of the ether extract (crude fat) of carrots containing cadmium, mercury and selenium proved to be lower (74–75%) than those of carrots containing lead (78%) and molybdenum (81%).

The final average body mass of the different experimental groups was practically identical. The feeding of carrots of high Cd concentration significantly decreased the (relative) mass of livers and hearts. The microelements differed as regards their accumulation in the organs. Mo and Cd accumulated in the kidneys,

Pb in the kidneys, liver, bones and lungs, while Hg in the kidneys and liver. Selenium was present practically in all soft tissues, i.e. in the liver, kidneys, testicles, ovaries and heart. For Mo and Se the main routes of elimination were the faeces and urine. In contrast, Cd, Pb and Hg were eliminated almost exclusively via the faeces. The retention rates were 19.82% for Mo, 62.14% for Cd, 33.61% for Pb, 35.35% for Hg, and 52.59% for Se.

Histopathologically, the testicles showed a reduced intensity of spermatogenesis in rabbits exposed to Mo, Cd, Pb and Hg. Disturbance of meiosis was detectable in the germinative tissue, with the presence of increased numbers of syncytial giant cells and other degenerated cells as compared to the control. The ovaries of female rabbits showed a normal histological picture (intact follicles at different phases of maturation, without corpora lutea) in the control group as well as in the Mo and Se groups. In the groups exposed to Cd, Pb and Hg a depression of follicle development was seen. Pb intake significantly decreased the activity of γ-glutamyltransferase (γ-GT) and increased that of ALP. Cd and Hg load also increased the activity of ALP. All experimental treatments decreased the cholinesterase activity of the blood. The activities of ALT, AST, CK and the level of creatinine practically did not change. In rabbits fed Mo-, Cd- and Se-containing carrots, glutathione peroxidase activity increased by 20–50%. Pb intake reduced the activity of glutathione peroxidase while Hg had practically no effect on it.

Fekete et al. (1994*b*) conducted a model experiment using cell cultures to predict the risks or benefits of cadmium for humans. The cell cultures were treated with a threefold dilution series of the test compound cadmium sulphate. The 50% cell multiplication inhibitory concentration (IC_{50}, mmol/l) was calculated by regression analysis. The IC_{50} values obtained for the different cell cultures allowed the authors to draw the following conclusions: (*i*) Cadmium abruptly inhibits cell multiplication and proliferation, without any transition. (*ii*) The IC_{50} values obtained for the different cell lines are in the same range (0.025–0.082 μmol/l). (*iii*) On the basis of the data obtained, cadmium can be considered a general cell toxicant.

Acknowledgement

This work was supported by the National Research Fund (OTKA), project no. 1270.

References

Ahles, J. and Rösick, E. (1986): Cd uptake and influence on plasma membrane function of yeast. Toxic. Environm. Chem. **11,** 291.

Ahokas, K. A., Dilts, P. V. and La Haye, E. B. (1980): Cd-induced fetal growth retardation: Protective effect of excess dietary Zn. Am. J. Obstetr. Gynecol. **132,** 216.

Alsberg, G. L. and Schwarze, E. W. (1919-1920): Some cases of testic degeneration. J. Pharm. Exp. Ther. **14,** 504.

Andresen, U. (1990): Untersuchungen zur Blei- und Cadmium Verteilung in verschiedenen Körperkompartimenten von Rindern und Kühen. Thesis, Hannover.

Anke, M. (1986): Mineralstoffe. In: Machhjolz, R. and Lewerenz, H. J. (eds): Lebensmitteltoxikologie. Akademieverlag, Berlin, 1986.

Anke, M., Hennig, A., Grün, M., Groppel, B. and Lüdke, H. (1981): In: Kirchgessner, M. et al. (eds) Trace Element Metabolism in Man and Animals **3,** 540. München-Weihenstephan.

Anke, M., Hennig, B., Groppel, B., Partschefeld, M. and Grün, M. (1978): The biochemical role of cadmium. In: Kirchgessner, M. (ed.) Trace Element Metabolism in Man and Animals. 3rd edn. Arbeitskreis Tierernährungsforschung, E. Weihenstephan, pp. 540–548.

Anke, M., Hennig, A., Grün, M., Groppel, B. and Lüdke, H. (1981): Cadmium and its influence on plants, animals and man with regard to geological and industrial conditions. Environm. **10,** 105.

Anke, M., Hennig, H. J., Schneider, H., Lüdke, H., Gargen, W. and Schlegel, H. (1970): In: Mills, C. F. (ed.) Trace Element Metabolism in Animals. Livingstone, Edinburgh and London, p. 317.

Anke, M., Hoffmann, G., Hennig, G., Groppel, B., Kronemann, H. and Grün, M. (1982): In: Anke, M. et al. (eds) Mengen und Spurenelemente. 2. 85., Wiss. Publ. Karl-Marx-Univ., Leipzig.

Anke, M., Klinger, G., Grün, M. and Schneider, H. J. (1977): The dependence of the cadmium concentration in animals and human beings on sex and age. In: Anke, M. et al. (1977) Cd 2. 72. Wiss. Publ. Karl-Marx-Univ., Leipzig.

Anke, M., Klinger, G. and Grün, M. (1982): The sex and age dependence of the cadmium concentration in human beings and animals. In: Anke, M. (ed.) Mengen and Spurenelemente. Karl-Marx-Universität, Leipzig. pp. 103–113.

Anke, M., Kronemann, H., Groppel, B. and Riedel, E. (1984): Die biologische Bedeutung kleinster Kadmiummengen für die Fauna. Wiss. Z. Karl.-Marx-Univ., Leipzig. Math. Naturwiss. R. **33,** 157–165.

Ashmead, H. D. and Jeppsen, B. R. (1993): Mineral amino acid chelates in nutrition. In: Anke, M. et al. (1993) Trace Elements in Man and Animals — Tema 8. Verl. Media Touristik, Jena, 1993, p. 341.

Bache, A. C., Lisk, J. D., Scarlett, M. J. and Carbone, G. (1991): Epidemiologic study of cadmium and lead in the hair of ceramists and dental personnel. J. Toxicol. Environm. Health **34,** 423–431.

Baird-Parker, A. C. and Davenport, E. (1965): The effect of recovery medium on the isolation of *Staphylococcus aureus* after heat treatment and after the storage of frozen or dried cells. J. Appl. Bacteriol. **28,** 390–402.

Baker, D. E., Amacher, M. C. and Learch, R. M. (1979): Sewage sludge as a source of cadmium in soil-plant-animal system. Environm. Health Perspect. **28,** 45–49.

Barcelo, J., Poschenrieder, Ch., Andreu, L. and Gunsé, B. (1986): Cd-induced decrease of water stress resistance in bush bean plants (*Phaseolus vulgaris* L.). J. Plant Physiol. **125,** 17.

Berg, D. and Kollmer, E. W. (1983): Absorption of cadmium from the intestine of the rat. Frequency distribution after a dose labelled by radio-activity during chronical infection. In: Heavy Metals in the Environment I. Internationale Konferenz. Heidelberg, pp. 553–556.

Berlin, M. and Ullberg, S. (1963): The fate of Cd 109 in the mouse. Health **7,** 686.

Black, F., Anke, M. and Schneider, H. J. (1979): Kadmium Symposium. Wissensch. Beitrage, Friedrich-Schiller Univ., Jena.

Block, M. and McBladt, C. (1991): Distribution of cadmium in an octanol/water system in the presence of xanthates and diethyldithiocarbamate. Environm. Toxicol. Chem. **10,** 1267–1272

Bokori, J. (1994): Occurrence in nature and main physiological properties of cadmium (in Hungarian, with English abstract). Magyar Állatorvosok Lapja **49,** 69–73.

Borgman, F. R. and Lightsey, F. S. (1982): Effects of synthesized hard water and of cadmium in the drinking water upon lipid metabolism and cholelithiasis in rabbits. Am. J. Vet. Res. **43,** 1432–1435.

Bruckner, Cl. (1988): Der Einfluss verschiedener Futterzusatzstoffe auf die Cadmium-Retention in der Niere beim Hühnerküken. Thesis, Munich.

Bundscherer, B. (1984): Der Einfluss der Zn-Zufuhr und verschiedener Bindungsformen des Cadmiums auf die Cadmium-Retention in Leber und Nieren beim Hühnerküken. Vet. Med. Diss., Munich.

Bunker, V. W., Lawsob, S. M., Belves, T. H. and Clayton, E. B. (1984): The intake and excretion of lead and cadmium by the elderly. Am. J. Clin. Nutr. **39,** 803–808.

Cadmium in Phosphates (1989): One part of a wider environmental problem. If it's a problem, what is the solution? Phosphorus and Potassium Congress no.162. July–August 1989, Michigan, U.S.A.

Chaney, R. L. and Hundemann, P. T. (1977): Nat. Conf. on Composting of Municipal Residues and Sludges. Inf. Transfer, Inc. Rockville, Maryland, U.S.A. p. 86.

Clare, E., Casey, E. C. and Robinson, F. M. (1978): Copper, manganese, zinc, nickel, cadmium and lead in human foetal tissues. Br. J. Nutr. **39,** 639.

Cole, J. and Ilzro, S. (1980): Cadmium occupational health research program. Cd Seminar. London, March 20, 1980.

Cook, J. A., Hoffmann, E. D. and Diluzio, N. R. (1975): Proc. Soc. Exp. Biol. Med. **150,** 741. In: Underwood, E. J. (ed.) (1977) Trace Elements in Human and Animal Nutrition. Academic Press, New York.

Cotzias, C. G., Borg, C. D. and Selleck, B. (1961): Specificity of the zinc pathway in the rabbit: zinc–cadmium exchange. Am. J. Physiol. **201,** 63–66.

Cousins, R. J. and Feldman, S. L. (1973): Effect of cholecalciferol on Cd-uptake in the chicken. Nutr. Rep. Internat. **8** (6) 363.

Davies, N. T. and Campbell, K. J. (1977): The effect of cadmium on intestinal copper absorption and binding in the rat. Life Sci. **20,** 955 –960.

Deudal, M. (1987): The effect of cadmium upon the structure of the olfactory nerve in the young pike. Toxicol. Environm. Chem. **14,** 227.

Doyle, J. J. and Pfander, W. H. (1975): Interactions of cadmium with copper, iron and manganese in ovine tissues. J. Nutr. **105,** 599.

Doyle, J. J., Pfander, W. H., Grebing, S. E. and Pierce, J. O. (1972): Effects of dietary cadmium on growth and tissue levels in sheep. In: Hemphill, D. D. (ed.) Trace Substances in Environmental Health. Univ. Missouri, Columbia **6,** 181–186.

Doyle, J. J., Pfander, W. H., Grebing, S. E. and Pierce, J. O. (1974): Effect of dietary cadmium on growth, Cd-absorption and Cd tissue levels in growing lambs. J. Nutr. **104,** 160.

Elsenhans, B., Schmolke, G., Jolb, K., Stokes, J. and Forth. W. (1987): Metal–metal interactions among dietary toxic and essential metals in the rat. Ecotoxicol. Environm. Safety **14,** 275–287.

Eybl, V., Kotyzova, D., Koutensky, J., Jones, M. M. and Sing, K. P. (1993): Influence of new dithiocarbamate analogs — BLDTC and MeBLDTC — on cadmium deposits and essential elements level in mice. In: Anke, M., Meissner, D. and Mills, C. F. (eds) Trace Elements in Man and Animals. TEMA 8. Verl. Media Touristic, Jena, 1933, p. 947.

Failla, M. N., Cousins, R. J. and Mascenk, M. J. (1979): Cadmium accumulation and metabolism by rat liver parenchymal cells in primary monolayer culture. Biochem. Biophys. Acta **543,** 63.

Fairchild, E. J., Lewis, R. J. and Tatkin, R. L. (1977): "Registry of Toxic Effects of Chemical Substances". DHEW Publ. No.78–104. B. Vol. 2. p.526. WSDHEW, Washington, D. C.

Fassett, D. W. (1972): Cadmium. In: Lee, D. H. K. (ed.) Metallic Contaminants and Human Health. Academic Press, New York. p. 97.

Favero, N., Costa, P. and Massimino, M. L. (1991): *In vitro* uptake of cadmium by *Bassidiomycetes pleurotus ostreatus*. Biotechnology Letters **13,** 701–704.

Fekete, S., Hullár, I., Kádár, I., Huszenicza, Gy., Glávits, R., Mézes, M. and Szilágyi, M. (1994*a*): Effects of the feeding of carrots, cultivated on fields polluted with Pb, Mo, Cd, Hg, Se, on digestion, reproduction and metabolism parameters of young rabbits. Proc. Soc. Nutr. Physiol. **2,** 57.

Fekete, S., Gálfi, P., Huszenicza, Gy. and Pais, I. (1994*b*): Study of the effect of cadmium upon cell cultures. Poster presented at the 6th Int. Trace Element Symp., Budapest, 30 June 1994.

Felley-Bosco, E. and Diezi, J. (1987): Fate of Cd in rat renal tubules: A microinjection study. Toxicol. Appl. Pharmacol. **91,** 204.

Fleischer, M., Sarofilm, W. D., Fassett, W. D. and Hammond, P. (1974*a*): Environmental impact of cadmium: A review by the panel on hazardous trace substances. Environm. Health Perspect. **5,** 253–311.

Fleischer, M., Sarofilm, A., Fassett, D. W., Hammond, P., Schacklette, T.H., Nisbet, T. C. J. and Epstein, S. (1974*b*): Environmental impact of cadmium. Environm. Health Perspect. Exp. Issue No. 253.

Flick, D. F., Kraybill, F. H. and Dimitroff, M. J. (1971): Toxic effects of cadmium: A review. Environm. Res. **4,** 71.

Fontana, A., Maggio, F. and Pizzino, T. (1987): On coordination models of adenine in Cd (II.1) complexes. J. Inorg. Biochem. **29,** 165.

Fox, M. R. S. (1974): Effect of essential minerals on cadmium toxicity. A review. J. Food Sci. **39,** 321–324.

Fox, M. R. S. (1979): Nutritional influences on metal toxicity: Cadmium as a model toxic element. Environm. Health Perspect. **29,** 95–104.

Fox, M. R. S. (1982): In: Prasad, S. A. (ed.) Clinical, Biochemical and Nutritional Aspects of Trace Elements. Fed. Am. Soc. Exp. Biol. Alan R.Liss.Inc, New York. p. 537.

Fox, M .R. S., Jacobs, M. R., Jones, L. O. and Frey, E. B. (1979): Effect of nutritional factors on metabolism of dietary cadmium at levels similar to those of man. Environm. Health Perspect. **28,** 107–114.

Fox, M .R. S., Tao, H., Stone, L. C. and Frey, E. B. (1984): Effects of zinc, iron and copper deficiencies on cadmium in tissues of Japanese quail. Environm. Health Perspect. **54,** 57.

Freeland, J. H. and Cousins, R. J. (1973): Effect of dietary cadmium on anaemia iron absorption and Cd binding protein in the chickens. Nutr. Rep. Internat. **8,** 337.

Friberg, L. (1950): Health hazards in the manufacture of alkaline accumulators with special reference to chronic cadmium poisoning. A clinical and experimental study. Acta Med. Scand. **138,** Suppl. 240, 1–124.

Friberg, L., Kjellström, T., Elinder, G. and Nordberg, G. F. (1985): Cadmium and Health: A Toxicological and Epidemiological Appraisal. Vol. 1. CRC Press, Boca Raton, Florida.

Friberg, L., Kjellström, T., Nordberg, G. F. and Piscator, M. (1975): Cadmium in the Environment III.: A Toxicological and Epidemiological Appraisal. Office of Research and Development, USEPA, Washington.

Friberg, L., Kjellström, T., Nordberg, G. F. and Piscator, M. (1979*a*): Cadmium. In: Friberg, L., Nordberg, G. F. and Vouk, V. (eds) Handbook on the Toxicology of Metals. Elsevier, Amsterdam. pp. 355–381.

Friberg, L., Nordberg, G. F. and Vouk, V. (1979*b*): Handbook on the Toxicology of Metals. Elsevier – North Holland Biomedical Press, Amsterdam – New York – Oxford.

Friberg, L., Piscator, M., Nordmerg, G. F. and Kjellström, T. (1974): Cadmium in the Environment. 2nd edn. Chem. Rubber Coop. Press, Cleveland, Ohio, U.S.A.

Frieden, E. (1985): New perspectives on the essential trace elements. J. Chem. Educ. **62,** 917.

Friel, J. K., Borgman, R. F. and Chandra, R. K. (1987): Effect of chronic Cd administration on liver and kidney concentrations of Zn, Cu, Fe, Mn and Cr. Bull. Envoiron. Contam. Toxicol. **38,** 588.

Furuta, H. (1977): Cd-effects on rat testicular polyamines. Toxic. Letters **1,** 141.

Gauthier, M. and Flatau, G. (1977): Concentration et mode de fixation du cadmium par un *Vibrio* marin. C. R. Acad. Sci. (Paris) **285,** 817–820.

Ghafghazi, T. and Mennear, J. H. (1975): The inhibitory effect of cadmium on the secretory activity of the isolated perfused rat pancreas. Toxic. Appl. Pharmacol. **31,** 134.

Glauser, S. C., Bello, C. T. and Glauser, E. M. (1976): Blood-Cd levels in normotensive and untreated hypertensive humans. Lancet **ii,** 717.

Godzik, S., Florkowsky, T., Plorek, S. and Sassen, A. N. M. (1979): An attempt to determine the tissue contamination of *Quercus robur* L. and *Pinus silvestris* L. foliage by particulates from Zn and Pb smelters. Environm. Pollut.**18,** 97–106.

Goering, P. L., Tandon, S. K. and Klaassen, C. D. (1985): Induction of hepatic metallothionein in mouse liver following administration of chelating agents. Toxic. Appl Pharmacol. **80,** 67.

Gomez, A., Desenfants, C. and Juste, C. (1988): "Transfer to cadmium du sol a la plante. Influence de la temperature du substrat et de la pesence d'autres metaux lourdes". C. R. Conv. Ministr. Environment France (INRA).

Groppel, B. (1969): Vergleichende Untersuchungen über den Mangan-, Zink- und Cadmiumstoffwechsel kleiner Wiederkäuer bei unterschiedlicher Versorgung mit diesen Elementen. Friedrich-Schiller-Univ., Jena. Math.-Naturwiss. Techn. Fac. Diss.

Groppel, B., Anke, M., Hennig, A. and Lüdke, H. (1979): In: Anke, M. and Schneider, H. J. (eds) Kadmium Symposium. Friedrich-Schiller-Univ., Jena.

Gruden, N. (1982): Cadmium–manganese interaction in the rat's intestinal tract. Heavy Metals in the Environment. Ist International Conference, Athen, pp. 676–678.

Gruden, N. and Munic, S. (1987): Effect of Fe upon Cd–Mn and Cd–Fe interaction. Bull. Environm. Contam. Toxicol. **38,** 969.

Gründer, H. D. (1982): Belastunggrenzen für Schwermetalle bei Hauswiederkauern. Landw. Forsch. **39,** 60–93.

Gunnarsson, O. (1983): Heavy metals in fertilizers: do they cause environmental and health problems? Fertilizers and Agriculture no. 85, September 1983, pp. 27–42.

Hansen, L. G. and Hinesly, T. D. (1979): Cadmium for soil amended with sewage sludge: effects and residues in swine. Environm. Health Perspect. **28,** 51–57.

Hapke, J. H. (1975): Toxikologie für Veterinärmediziner. Ferdinand Enke Verlag, Stuttgart.

Hapke, J. H. (1992): Auf Tiere einwirkende toxische Umweltnoxen. Züchtungskunde **64,** 262–270.

Hapke, J. H., Abel, J., Kühl, U. and Glaser, U. (1977): Die Amsamlung von Cadmium in verzehrbaren Geweben in Abhängigkeit von der Cadmium-Menge im Futter. Arch. Lebensm. Hyg. **28,** 174–177.

Hecht, H. (1979): Zum carry over von toxischen Elementen in das von Nutztieren. Fleschwirtsch. **59,** 1621–1629.

Hecht, H. (1983): Untersuchungen über endogene und exogene Einflüsse Blei-und Cadmiumgehalt in Muskel- und Organproben von Rehwild; der Einfluss von alter und Versuchsort. Abschlussbericht,Vorhaben Ha517/20 und Ha517/24 der Deutschen Forschungsgemeinschaft. Teil I, Kulmbach.

Hecht, H. (1986): Unterschiede der Gehalte an Spurenelementen in Muskeln und Organen von Wild- und landwirtschaftlichen Nutztieren. Fleischwirtsch. **66,** 1.

Hoffmann, G., Anke, G., Pertschefeld, M., Groppel, B. and Kronemann, H. (1979): In: Anke, M. and Schneider, H. J. (eds) Cadmium-Symposium. Friedrich-Schiller Univ., Jena. pp. 58–62.

Hogan, G. R. and Jackson, P. D. (1986): Dichotomous effects of Cd and Se on erythropoiesis in mice. Bull. Environm. Contam. Toxicol. **36,** 674.

Holcman, A. and Smodis, B. (1993): Heavy metal content in hen's eggs. In: Anke, M., Meissner, D. and Mills, F. C. (eds) Trace Elements in Man and Animals. Tema 8. Verlag Media Touristic, Jena 11993, 249.

Holden, H. (1980): Cadmium and pulmonary emphysema. Lancet **i** (8178), 1137.

Holt, D. and Webb, M. (1987): Teratogenicity of ionic cadmium in the Wistar rat. Arch. Toxicol. **59,** 443.

Horowitz, C. T. (1988): Is the major part of the periodic system realy inessential for life? J. Trace Element Electrolyte Hlth. Diss. **2** (3) 135-144.

Itokawa, Y., Abe, T., Tabei, R. and Tanak, S. (1974): Renal and skeletal lesions in experimental cadmium poisoning. Arch. Environm. Health **28,** 149.

Jacobs, R. M., Fox, M. R. S., Fry, E. B. and Harland, F. B. (1974*a*): The effect of two day exposure to dietary cadmium on the concentration of elements in tissues of Japanese quail. In: Hoekstra, W. G., Suttle, W. J., Ganther, E. H. and Mertz, W. (eds) Trace Element Metabolism in Animals 2. Park Press, Baltimore, Maryland. pp. 684–686.

Jacobs, R. M., Fox, S. R. M., Lee, O. A., Harland, F. B. and Fry, E. B. (1974*b*): Increased duodenal and decreased hepatic and renal Cd in Japanese quail fed supplements of Zn, Mn and Cu. Fed. Proc. **33,** 668.

Jacobs. R. M., Jones, A. O. L., Fox, M. R. S. and Fry, E. B. (1978): J. Nutr. **108,** 22. In: Underwood, E. J. (ed.) (1977): Trace Elements in Human and Animal Nutrition. Academic Press, New York.

Jacobs, R. M., Jones, A. O., Fox, M. R. S. and Lener, J. (1983): Effects of dietary zinc, mangenese and copper on tissue accumulation of cadmium by Japanese quail. Proc. Soc. Exp. Biol.Med. **172,** 34–38.

Johnson, D. E., Prevost, R. J., Tillery, J. B. and Thomas, R. E. (1977): Final Report Contract no. 68-02-1725. Prepared by Southwest Research Institute, San Antonio, Texas for USEPA. Washington, D. C.

Jones, K. C. and Johnston, A. E. (1989): Cadmium in cereals and herbage from long-term experimantal plots at Rothamsted, U.K. Environmental Pollution **57,** 199–216.

Jopek, Z., Kaszubrkiewicz, C. and Madej, A. (1980): Über den Gehalt der Schwermetalle Zn, Cu, Cd, Pb in den Organen Lympholeukotischer Kühe. Arch. Exp. Vet. Med. **34,** 663–668.

Juste, C. and Mench, M. (1992): Long-term application of sewage sludge and its effects on metal uptake by crops. In: Domy, C. A. (ed.) Biogeochemistry of Trace Metals. Lewis Publishers, London–Tokyo.

Kar, A. B., Das, P. R. and Mukerji, I. N. F. (1960): Prevention of cadmium-induced changes in the gonads of rats by zinc and selenium. Proc. Natl. Inst. Sci. Indiana **26,** 40–50.

Kádár, I. (1991): Research on Environmental Protection and Nature Conservation (in Hungarian). Akaprint Nyomdaipari Kft., Budapest.

Kelman, B. J. and Walter, B. K. (1977): Passage of Cd across the perfused guinea pig placenta. Proc. Soc. Exp. Biol. **156,** 68.

Kleiminger, B. J. (1983): Untersuchungen über die Eignung von freilebenden Wildarten als Bioindikatoren zur Erfassung von fachenhaften Schwemetallkontaminationen in Niedersachsen. DVM Thesis, Hannover, 1983.

Koller, L. D., Exon, J. H. and Raan, J. G. (1975): Antibody suppression by cadmium. Arch.Environm. Health **30,** 598.

Kollmer, W. E. and Berg, D. (1988): The influence of zinc-calcium or iron-deficient diet on the resorption and kinetics of cadmium in the rat. Proceedings of the Congress on Bioavaility **88,** 112.

Korkeala, H. (1980): The effect of vitamin E on the toxicity of cadmium in cadmium-sensitive *Staphylococcus aureus*. Acta Vet. Scand. **21,** 224–228.

Korkeala, H. and Sankari, S. (1980): The effect of catalase on the toxicity of cadmium in cadmium-sensitive and cadmium-resistant *Staphylococcus aureus*. Acta Vet. Scand. **21,** 209–223.

Korkeala, H., Uusi-Rauva, A. and Pekkanen, J. T. (1981): The effect of cysteine and sodium selenite on the toxicity of cadmium in *Staphylococcus aureus*. Acta Vet. Scand. **22,** 129–136.

Kosla, T., Roga-Franc, M., Rokicki, E. and Palutek, T. (1993): The effect of cadmium contamination on serum levels in mineral elements in dairy cows. In: Anke, M., Meissner, D. and Mills, F. C.: Trace Elements in Man and Animals. Tema 8. Verl. Media Touristik, Jena, 1993. p. 329.

Kovács, F. and Ványi, A. (1993): Movement of certain heavy metals, nitrates and mycotoxins in the food chain (in Hungarian, with English abstract). Magyar Állatorvosok Lapja **48,** 197–201.

Kozlowska, K., Brzozowska, A., Sulkowska, J. and Roskowski, W. (1993): The effect of cadmium supplied orally on iron, zinc and copper in rat tissues. In: Anke, M., Meissner, D. and Mills, C. F. (eds) Trace Elements in Man and Animals. Tema 8. Verl. Media Touristik, Jena, 1993. p. 581.

Köfer, J. and Fuchs, K. (1993): Rückstandmonitoring bei Fleisch. 2. Mitteilung: Umweltkontaminanten (Pb, Cd) in Rindernieren. Wiener Tierärztl.Mschr. **80,** 264–267.

Kranjc, E. I., Vos, J. G. and Logtent, M. J. (1983): In: Health Evaluation of Heavy Metals in Infant Formula and Junior Food. Springer-Verlag, Berlin – New York.

Kramer, K., Anke, M., Glei, M. and Müller, M. (1993): Effects of oral bentonite offers on macro and trace element incorporation of different parts of the body and the milk. In: Anke, M., Meissner, D. and Mills, F. C. (eds) Trace Elements in Man and Animals. Tema 8. Verlag Media Touristik, Jena, 1993.

Kreuzer, W. (1983): Schwermetalle in Fleisch und Organen von Schlachttieren Vortrag anlassl. des 11. Seminares Umwelthyg. Hannover, 1983.

Kreuzer, W. (1986): Zum Übergang von Cadmium bei Mast- und Nutzrindern. In: Zum carry over von cadmium. Cadmiumkontamination von Futtermitteln und Auswirkungen auf die Tierische Erzeugung. Schriftenr. Bundesminist. **335,** 57–72. Landwirtschaftsverlag GmbH, Münster.

Kreuzer, W., Bunzi, K. and Kracke, W. (1981): Zum Übergang von Cadmium aus dem Futter in Nieren, Lebern und Musculatur von Schlachtrindern. Fleischwirtsch. **61,** 1886.

Kreuzer, W. and Rosopulo, A. (1981): Zur gegenwärtigen Rückstandssituation bei Cadmium, Blei, Quicksilber und Arsen in Fleisch und Organen von Schlachttieren. Arch. für Lebensmittel Hyg. **32,** 181.

Kronemann, H., Anke, M. and Grün, M. (1982): Kadmiumgehalt des Deckhaares der Milchkühe in Abhängigkeit von der geologischen Herkunft des Standartes und Kadmiumemissien. In: Anke, M. (ed.) Mengen und Spurenelementen. Karl-Marx-Univ., Leipzig, 115.

Krzystyniak, K., Fournier, M., Trottier, B., Nadeau, D. and Chevalier, G. (1987): Immunosuppression in mice after inhalation of Cd. Toxicology Letters **38,** 1.

Kucharz, E. I. (1988): Effect of Cd-intoxication on collagen and elastin content in tissues of the rat. Bull. Environm. Contam. Toxicol. **40,** 273.

Kühl, U. U. (1976): Toxikologische und biochemische Untersuchungen zur Feststellung subklinischer Cadmiumwirkungen bei Schafen. Thesis, Tierärztl. Hochschule, Hannover.

Learch, R. M., Wang, L. W. K. and Baker, D. E. (1979): Cadmium and the Foodchain. The effect of dietary Cd on tissue composition in chicks and laying hens. J. Nutr. **109,** 437–443.

Liebig, R. G. M. (1913): Zink und Cadmium. Verlag Otto Spanner, Leipzig.

Lindner, J. (1989): Untersuchungen über endogene und exogene Einflüsse auf die Cadmium-, Blei-, Quecksilber-, Kupfer- und Zinkgehalte in Organen und Muskulatur von Feldhasen. Thesis, Giessen.

Lodenius, M., Kuusi Laaksovirta, K., Lilja, H. and Piepponen, S. (1981): Lead, cadmium and mercury contents of fungi in Mikkeli, S. E. Finland. Am. Bot. Fennici **18,** 183.

Lopez, A. and Williams, H. L. (1985): Essential elements and Cd and Pb in fresh, canned and frozen green beans (*Phaseolus vulgaris*). J. Food Sci. **50,** 1152.

Lorentzon, R. and Larsson, S. E. (1977): Clin. Sci. Mol. Med. **53,** 439. In: Underwood, E. J. (ed.) (1977): Trace Elements in Human and Animal Nutrition. Academic Press, New York.

Machelett, B. and Bergmann, H. (1993): Heavy metal accumulation of plants depending on the pH-value and clay content of soil. In: Anke, M., Meissner, D. and Mills, F. C. (eds) Trace Elements in Man and Animals. Tema 8. Verl Media Touristik, Jena, 1993. p. 484.

Mahaffey, K. R., Corneliussen, P. E., Jelenek, P. E. and Fiorino, J. A. (1977): Environm. Health Perspect. **12,** 63. In: Underwood, E. J. (ed.) (1977) Trace Elements in Human and Animal Nutrition. Academic Press, New York.

Marletta, P. P., Favretto, L. G. and Favretto, L. (1986): Cd in roadside grapes. J. Sci. Food Agric. **37,** 1091.

Mason, K. E., Richardson, M. E. and Fox, M. R. S. (1977): Fed. Proc. Fed. Am. Cos. Exp. Biol. **36,** 1152. In: Underwood, E. J. (ed.) Trace Elements in Human and Animal Nutrition. Academic Press, New York.

May, P. M. and Bulman, R. A. (1983): The present status of chelating agents in medicine chemistry. Progr. Med. Chem. **20,** 225.

Menden, E. E., Ella, J. V., Michael, W. L. and Petering, G. H. (1972): Distribution of cadmium and nickel of tobacco during cigarette smoking. Environm. Sci. Technol. **6,** 830–832.

Mertz, W. (1986): Trace Elements in Human and Animal Nutrition. Fifth Edition. Academic Press, New York.

Miller, W. J., Blackmon, M. D., Gentry, P. R. and Pate, M. F. (1969): Effect of dietary cadmium on tissue distribution of ^{109}Cd following a single dose in young goats. J. Dairy Sci. **52,** 2029–2035.

Miller, W. J., Blackmon, M. D. and Martin, G. Y. (1968): ^{109}cadmium absorption, excretion, and tissue distribution following single tracer oral and intravenous doses in young goats. J. Dairy Sci. **51,** 1836–1839.

Mills, C. F. (1974): Trace element interactions: Effect of dietary compsosition on the development of imbalance and toxicity. In: Hoekstra, W. G., Suttle, W. J., Ganther, E. H. and Mertz, W. (eds) Trace Element Metabolism in Animals 2. University Park Press, Baltimore. pp. 79–90.

Mills, C. F. and Delgarno, A. C. (1972): Copper and zinc status of ewes and lambs receiving increased dietary concentrations of cadmium. Nature **239,** 171.

Mineral Tolerance of Domestic Animals. National Academy of Sciences, Washington, D.C., 1980.

Mitani, T. and Hara, K. (1991): Characteristics of cadmium accumulation by batch activated sludge. Nippon Nogeikagaku Kaishi **65** (11) 1643–1646.

Moore, W., Stara, F. J., Crocker, C. W., Malachuk, M. and Iltis, R. (1973): Comparison of 115cadmium retention in rats following different routes of administration. Environm. Res. **6,** 473.

Moreau, T., Lellouch, B., Jugnet, B., Festy, B., Orssaud, G. and Claude, R. J. (1983): Cigarettes as a source of some trace and heavy metals and pesticides in man. Arch. Environm. Health **38,** 163–167.

Morgan, R. M., Kundomal, Y. R. and Hupp, E. W. (1985): Serum creatine kinase (CK) activity following exposure to Cd and or ^{60}Co gamma irradiation. J. Environm. Sci. Health A **20,** 681.

Mukherjee, A., Sharma, A. and Talukder, T. (1988): Effect of selenium on cadmium-induced chromosomal aberrations in bone marrow cells in mice. Toxicology Letters **41,** 23–29.

Neathery, M. W., Miller, W. J., Gentry, P. R., Stake, E. P. and Blackmon, M. D. (1974): Cd-109 and methylmercury-208 metabolism, tissue distribution and secretion into milk of cows. J. Dairy Sci. **57,** 1177–1188.

Nielsen, G. and Bjerregaard, P. (1991): Interaction between accumulation of cadmium and selenium in the tissues of turbot *Scophthalmus maximus*. Aquat. Toxicol. **20,** 253–266.

Noever, A. D. and Matsos, C. H. (1991): Calcium protection from cadmium poisoning. Environm. Sci. Health A **26,** 1105–1113.

Noever, A. D., Matsos, C. H. and Looger, L. L. (1992): Bioconvective indicators in *Tetrahymena*: Nickel and copper protection from cadmium poisoning. J. Environm. Sci.Health A **27,** 403–417.

Nolan, C. V. and Shaikh, Z. A. (1986): Determination of metallothionein in tissues by radioimmunoassay and by Cd saturation method. Anal. Biochem. **154,** 213.

Nomiyama, K., Sato, C. and Yamamoto, A. (1973): Early signs of Cd intoxication in rabbits. Tox. Appl. Pharmacol. **24,** 625.

Nordberg, G. F. (1975): Effect of long-term Cd exposure on the seminal vesicles of mice. J. Reprod. Fertil. **45,** 165.

Nordberg, G. F., Kjellström, T. and Nordberg, M. (1985): Kinetic and metabolism. In: Cadmium and Health: A Toxicological and Epidemiological Appraisal, Vol. I. CRC. Press, Boca Raton, Florida, U.S.A.

Oberdörster, G., Oldiges, H. and Zimmermann, B. (1980): Lung deposition and clearance of cadmium in rats exposed by inhalation or by intratracheal instillation. Zbl. f. Bakteriologie (B) **170,** 35–43.

Ohta, H., Seky, Y., Imamiya, S. and Yoshikawa, H. (1993): Influence of dietary selenium (Se) on chronic cadmium (Cd) toxicity in mice. In: Anke, M., Meissner, D. and Mills, C. F. (eds) Trace Elements in Man and Animals. Tema 8. Verlag Media Touristik, Jena, 1993. p. 963.

Ohanian, V. E., Iwai, J., Leitl, G. and Tuthill, R. (1978): Genetic influence on cadmium-induced hypertension. Am. J. Physiol. **235,** 385.

Pacyna, J. M. (1983): The atmospheric distribution of trace elements from anthropogenic sources in Europe. In: Heavy Metals in the Environment I. International Konferenz. Heilderberg, **178,** 181.

Pais, I. (1980): Role of Micronutrients in Agriculture (in Hungarian). Mezőgazdasági Kiadó, Budapest.

Pais, I. (1989): Importance of Microelements for Life (in Hungarian). University of Horticulture and Food Industry, Budapest.

Parizek, J. (1983): In: Clarcson, T. W., Nordberg, F. G. and Sager, R. P. (eds) Reproductive and Developmental Toxicity of Metals. Plenum Press, New York. p. 301.

Parizek, J., Ostadalova, I., Benes, I. and Babocky, A. (1968): Pregnancy and trace elements: The protective effect of compounds of an essential trace element, selenium against the peculiar toxic effects of cadmium during pregnancy. J. Reprod. Fertil. **16,** 507–509.

Parizek, J. and Zahor, Z. (1956): Effect of cadmium salts on testicular tissue. Nature **177,** 1036.

Perry, H. M., Erlanger, M. and Perry, E. F. (1977): Elevated systolic pressure following chronic feeding. Am. J. Physiol. **232,** 114.

Perry, H. M. and Perry, E. F. (1974): Possible relationship between the physical environment and human hypertension: Cadmium and hard water. Prev. Medicine **3,** 344–352.

Perry, H. M., Thinds, S. and Perry, E. F. (1976): The biology of cadmium. Medical Clinics of North America **60,** 759–769.

Petering, H. G., Chowdhury, H. and Stemmer, L. K. (1979): Some effects of oral ingestion of cadmium on zinc, copper and iron metabolism. Environm. Health Perspect. **28,** 97–106.

Piscator, M. (1974): Cadmium in the Environment. 2nd edition. CRC.Press, Cleveland, Ohio, U.S.A., p. 71.

Piscator, M. (1982): In: Prasad, S. A. (ed.) Clinical, Biochemical and Nutritional Aspects of Trace Elements. Alan R. Liss, Inc., New York. p. 521.

Piscator, M. (1983): In: Health Evaulation of Heavy Metals in Infant Formula and Junior Food. Springer-Verlag, Berlin – New York.

Posthuma, L., Hogervorst, F. R. and Straalen, M. N. (1992): Adaptation to soil-pollution by cadmium excretion in natural populations of *Orhesella cincta* L. (*Collem bola*). Arch. Environm. Contam. Toxicol. **22,** 146–156.

Powell, G. W., Miller, W. J., Morton, D. J. and Clifton, C. M. (1964): Influence of dietary cadmium level and supplemental Zn on cadmium toxicity in the bovine. J. Nutr. **84,** 205.

Pribble, H. J. (1973): Effects of aqueous and dietary Cd on rat growth and tissue uptake. Bull. Environm. Cont.Toxic. **9,** 271.

Pritzl, C. M., Lie, H. Y., Kienholz, W. E. and Whiteman, E. C. (1974): The effect of dietary cadmium on development of young chickens. Poultry Sci. **53,** 2026–2029.

Rabar, I. and Kostial, K. (1981): Bioavailability of cadmium in rats fed farious diet. Arch. Toxicol. **47,** 63–66.

Rahola, T., Aaran, K. R. and Miettinen, K. J. (1972): Half-time studies of mercury and cadmium by whole-body counting. In: Assessment of Radioactive Contamination in Man. Proceeding Series, International Atomic Energy Agency. Unipublishers, New York **13,** 553–562.

Rambeck, A. W., Brehm, W. H. and Kollmer, E. W. (1991): Der Einfluss erhöhter Kupferzulagen zum Futter auf die rückstandbildung von Cadmium bei Schwein. Zschr. f. Ernährungswissenschaft **30,** 299–300.

Régius-Mőcsényi, Á. (1991): Zinc, manganese, copper, molybdenum, nickel and cadmium supply status of cattle, sheep and horses. 6th part: Cadmium supply status (in Hungarian). Állattenyésztés és Takarmányozás **40,** 465–477.

Régius-Mőcsényi, Á., Anke, M., Kronemann, H. and Szentmihályi, S. (1985): Cadmium content and influence of cadmium contamination in plants and animals. In: Pais, I. (ed.) New Results in the Research of Hardly Known Trace Elements. Proc. Internat. Symp. Univ. Horticult., Budapest.

Richardson, M. E. and Fox, M. R. S. (1974): Dietary cadmium and enteropathy in the Japanese quail: Histochemical and ultrastructural studies. Lab. Invest. **31,** 722–731.

Roels, H. A. and Lauwerys, R. R. (1983): In: Schmidt, F. H. and Hildebrandt, G. A. (eds) Health Evaluation of Heavy Metals in Infant Formula and Junior Food. Springer-Verlag, Berlin – New York. p. 126.

Rudd, C. J. and Herschman, H. R. (1979): Metallothionein in human cell line. The response of HeLa to Cd and Zn. Toxic. Appl. Pharmacol. **47,** 273.

Rude, N. (1982): Transfer of cadmium through the rat's intestinal wall. Environm. Res. **28,** 340–343.

Runkel, K. H. and Payer, D. H. (1984): Resorption von Cadmium und Zink aus kontaminierten Futtermitteln und Akkumulation von Cadmium in Leber und Niere. GSF Bericht **A6,** 84.

Ryan, J. A., Pahren, H. R. and Lucas, J. B. (1982): Controlling cadmium in the human food-chain: a review and nationale based on health effects. Environm. Res. **28,** 251.

Sanders, C. L. and Mahaffey, J. A. (1984): Environm. Res. **33,** 227. In: Mertz, W. (ed.) (1986) Trace Elements in Human and Animal Nutrition. Fifth Edition. Academic Press, New York.

Sandstead, H. H., Doherty, R. A. and Mahaffey, J. A. (1983): In: Clarcson, T. W., Nordberg, G. F. and Sager, R. P. (eds) Reproductive and Development Toxicity of Metals. Plenum Press, New York, p. 205.

Sarhan, M. J., Roels, H., Lauweris, R., Reyners, H. and Gianfelici de Reyners, E. (1986): Influence of manganese on the gastrointestinal absorption of cadmium in rats. Appl. Toxicol. **6,** 313.

Sas, B. and Tu, P. V. (1989): Detection and effects of food-contaminating toxic elements, with especial regard to some toxicological and biochemical consequences of cadmium load (in Hungarian). Publication of the Environmental Protection Conference of the Hungarian Association for Agricultural Science, Budapest, 1989.

Schenkel, H. (1983): Einflussfaktoren auf das Stoffwechselverhalten von Schwermetallen bei landwirtschaftlichen Nutztieren. Wissenschaft und Umwelt **4,** 289–295.

Schenkel, H. (1986): Zum Einfluss verschiedener Nahrungsfaktoren auf das Stoffwechselverhalten von Cadmium bei landwirtschaftlichen Nutztieren. In: Zum Carry-over von Cadmium. Schriftenreihe des Bundesministers für Ernährung, Landwirtschaft und Forsten. Reihe A, Heft **335,** 137–158.

Schenkel, H. (1988): Zum Stoffwechselverhalten von Cadmium bei landwirtschaftlichen Nutztieren. I.Mitteilung: Rinder. Übers.Tierernährung **16,** 265–306.

Schenkel, H. (1990): Zum Stoffwechselverhalten von Cadmium bei landwirtschaftlichen Nutztieren. II. Mitteilung: Kleine Wiederkauer und Schweine. Übers. Tierernährung **18,** 39–76.

Schinner, W. (1981): Untersuchung über endogene und exogene Einflüsse auf den Blei- und Cadmiumgehalt in Muskeln und Organen von Rehwild und Wildkaninchen. Thesis, Munich.

Schramel, P., Masse, S. and Ovcar-Pavlu, J. (1988): Se, Cd, Pb and Hg concentrations in human breast milk, in placentas, maternal blood of the newborn. Biol. Trace Element Res. **15,** 111.

Schroeder, H. A. and Balassa, J. J. (1961): Abnormal trace metals in man: Cadmium. J. Chron. Dis.**14,** 236.

Schroeder, H. A., Balassa, J. J. and Vinton, W. H. (1965): Chromium, cadmium and lead in rats: Effect of life span. J. Nutr. **86,** 51.

Schroeder, H. A. and Mitchener, M. (1971): Arch. Environm. Health 23-102. In: Mertz, W. (ed.) (1986): Trace Elements in Human and Animal Nutrition. 5th edition. Academic Press, New York.

Schwarz, Th., Busch, A. and Lenk, R. (1991): Erste Untersuchungen zur Belastung von Futtermitteln, Rindern und Lebensmitteln tierischer Herkunft aus unterschiedlichen Produktionsgebieten Sachsens mit Blei, Kadmium un Arsen. Tierärztl.Wschr. **98,** 365–404

Schwarz, K. and Spallholz, J. E. (1979): The potential essentiality of cadmium. In: Anke, M. and Schneider, H. J. (eds) Kadmium-Symposium **2,** 188–194. Friedrich-Schiller Univ., Jena.

Scott, R. and Aughey, E. (1979): Brit. J. Urol. **50,** 25. In: Mertz, W. (ed.) (1986): Trace Elements in Human and Animal Nutrition. 5th edition. Academic Press, New York.

Scott, R., Aughey, E., Fell, G. S. and Quinn, M. J. (1987): Cadmium concentrations in human kidneys from the UK. Human Toxicol. **6,** 111.

Sharma, P. R., Street, C. J., Verma, P. M. and Shupe, K. L. J. (1979): Cadmium uptake from feed and its distribution to food products of livestock. Environm. Health Perspect. **28,** 59–66.

Sittig, M. (1976): Toxic Metals, Pollution Control and Worker Protection. Noyes Data Corporation, New Jersey, U.S.A.

Smith, M. R., Griel, C. L., Müller, D. L., Leach, M. R. and Baker, E. D. (1991*a*): Effects of dietary cadmium chloride throughout gestation on blood of cattle. J. Anim. Sci. **69,** 4078–4087.

Smith, M. R., Leach, M. R., Müller, D. L., Griel, C. L. and Baker, E. D. (1991*b*): Effect of long-term dietary cadmium chloride on tissue, milk and urine mineral concentrations of lactating dairy cows. J. Anim. Sci. **69,** 4088–4096.

Sonavane, B. R., Nordberg, G. M., Nordberg, G. F. and Lucier, W. G. (1975): Placental transfer of cadmium in rats.:Influence of dose and gestational age. Environm. Health Perspect. **12,** 97.

Stadelman, J. W. and Prati, E. D. (1989): World's Poultry Science Journal **45,** 247–246. In: Anke, M., Meissner, D. and Mills, F. C. (eds): Trace Elements in Man and Animals. Tema 8. Verl. Media Touristik, Jena.

Stolley, U. (1989): Der Einfluss verschiedener Vitamine und Spurenelemente auf die Cadmium-retention in der Niere beim Hühnerküken. Thesis, Munich.

Sturkie, P. D. (1973): Effect of Cd on electrocardiogram, blood ddpressure, and hematocrit of chickens. Avian Dis. **17,** 106.

Sugawara, N. and Sugawara, C. (1975): Effect of cadmium *in vivo* and *in vitro* on intestinal brush border ALPase and ATPase. Bull. Environm. Contam. Toxicol. **14,** 653.

Sumino, K., Hayakawa, K., Shibata, T. and Kitamura, S. (1975): Heavy metals in normal Japanese quail tissues. Arch. Environm. Health **30,** 487–507.

Szymanska, E. and Laskowska-Klita, T. (1993): Effect of cadmium ($CdCl_2$) on lipid peroxidation and activities of antioxidant enzymes in villus and crypt cells of rat small intestine. In: Anke, M., Meissner, D. and Mills, C. F. (eds) (1993) Trace Elements in Man and Animals. Tema 8. Verlag Media Touristik, Jena. p. 587.

Tahvonen, R. and Kumpulainen, J. (1993): Lead and cadmium contents in meat and liver produced in Finland in 1991. In: Anke, M., Meissner, D. and Mills, F. C. (eds): Trace Elements in Man and Animals. Tema 8.Verlag Media Touristik, Jena. p. 502.

Takenaka, S., Oldiges, H., Konig, H., Hochrainer, D. and Oberdoerster, G. (1983): JNCL J. Natl. Cancer.Inst. **70,** 367.

Takeuchi, J. (1973): The etiology of "Itai-Itai" disease. Jpn. J. Clin. Med. **81,** 132.

Tanaka, K., Min, K. S., Ohianagi, J., Onosaka, S. and Fukuhara, C. (1986): Fate of erythrocyte Cd-metallothionein in mice. Toxic. Appl. Pharmac. **83,** 197.

Tanaka, K., Sueda, K., Onosaka, S. and Okahara, K. (1975): Fate of ^{109}Cd-labelled metallothionein in rats. Toxic. Appl. Pharmacol. **33,** 258.

TECMIN (1984): The Cadmium Question: A Survey. TECMIN, Paris, 1984.

The Task Group on Metal Interaction (1978): Factors influencing metabolism and toxicity of metals: A consensus report. Environm. Health Perspect. **25,** 347.

Tohyama, C., Kobayashi, E., Saito, H., Sugihara, N., Nakano, A. and Mitane, Y. (1986): Urinary alpha-1 microglobulin as an indicator protein of renal tubular dysfunction caused by environmental cadmium exposure. J. Appl. Toxicol. **6,** 171.

Toluczo, J. (1989): If it's a problem, what is the solution? Cadmium in phosphates: one part of a wider environmental problem. Phosphorus and potassium. No. 162. July–August 1989, pp. 23–30.

Tu, P. V. (1989): Food toxicological importance of the cadmium content of animal tissues (in Hungarian). Thesis, Budapest.

Tynecyca, Z. J., Zajac, J. and Gos, Z. (1975): Plasmid dependent inpermeability barrier to cadmium ions in *Staphylococcus aureus*. Acta Microbiol. Pol. Ser. A **7,** 11–20.

Underwood, E. J. (1977): Trace Elements in Human and Animal Nutrition. Academic Press, New York.

Vaessen, H. A. M. and Fellen, G. (1986): Arseen, cadmium, kwik, lood en seleen inschlachtdiener: een overzücht jaar onderzoek. Tijdschr. Diergeneesk. **14,** 111.

Van Bruwaene, R., Gepber, G. B. and Fishman, R. (1982): Transfer and distribution of radioactive cadmium in dairy cows. Int. J. Environm. Scotland **18,** 42–51.

Van Bruwaene, R. and Kirchman, R. (1979): Transfer and distribution of cadmium in cows In: Int. Cond. Management and Control of Heavy Metals in the Environment, Edinburgh, pp. 39-48.

Varo, P., Lâhelma, O., Nuurtamo, M., Sari, E. and Koiviostoinen, P. (1980): Mineral element composition of Finnish foods. III. Annual variations in the mineral element composition of cereal grains. Acta Agric. Scand. **22,** 27–35.

Ward, N. I. (1993): The of cadmium intake from smoking activity (non, active and passive) on the outcome of pregnancy. In: Anke, M., Meissner, D. and Mill, C. F. (eds) Trace Elements in Man and Animals. Tema 8. Verlag Media Touristik, Jena, 1993. p. 872.

Washko, P. H. and Cousins, J. R. (1976): Metabolism of ^{109}Cd in rats fed normal and low-calcium diets. J. Toxic. Environm. Health **1,** 1055–1066.

Weigel H. J., Elmadfa, I. and Jager, H. J. (1987): Availability and toxicological effects of levels of biologically bound Cd. Arch. Environm. Contam. Toxicol. **16,** 85.

Weigert, P., Müller, J., Klein, H., Zufelde, P. and Hillebrand, J. (1984): Arsen, Blei, Cadmium und Quecksilber in und auf Lebensmitteln. ZEBS-Hefte **1,** 31 and 67.

Whanger, P. D. (1973): Effect of dietary Cd on intracellular distribution of hepatic Fe in rats. Res. Comm. Chem. Path. Pharmacol. **6,** 733.

WHO (1979): Environmental Health Criteria for Cadmium, Mercury and Silver. Interim Report, EHE/EHC 79.29, Geneva.

Wojciechowska-Mazurek, M. K., Starska, K., Zawadska, T., Karlowski, K., Brulinska-Ostrowska, M. (1993): Cadmium, copper, lead and zinc content in cereals and their products from different regions of Poland. In: Anke, M., Meissner, D. and Mills, C. F. (eds) Trace Elements in Man and Animals. Tema 8. Verlag Media Touristik, Jena, 1993. p. 261.

Wong, P. K. (1988): Mutagenicity of heavy metals. Bull. Environm. Contam. Toxicol. **40,** 597.

Wright, F. C., Palmer, M. J. S., Riner, J. C., Hanfler, N., Miller, J. A. and McBeth, C. A. (1977): J. Agric. Food Chem. **25,** 293. In: Underwood, E. J. (ed.) (1977): Trace Elements in Human and Animal Nutrition. Academic Press, New York.

Yayawickreme, C. K. and Chatt, A. (1990): Studies on zinc- and cadmium-bound proteins in bovine kidneys by biochemical and neutron activation techniques. Biol. Trace Element Res. **26-27,** 503-512.

Yoshida, S. (1986): Isolation and partial characterisation of a Cd-binding protein in soy beans from a Cd-pulleted field. Agric. Biol. Chem. **50,** 2273.

Yoshizuka, M., McCarthy, K. J., Kaye, G. I. and Fujimoto, S. (1990): Cadmium toxicity to the cornea of pregnant rats. Electron microscopy and X-ray microanalysis. Anat. Res. **227,** 138–143.

Yoshizuka, M., Mori, N., Hamasaki, K., Tanaka, I., Yokoyama, M., Hara, K., Doi, Y., Umezu, Y., Araki, H., Sakamoto, Y., Miyazaki, M. and Fujimoto, S. (1991): Cadmium toxicity in the thyroid gland of pregnant rats. Exp. Mol. Pathol. **55,** 97–104.

Acta Veterinaria Hungarica 43 (1), pp. 45–62 (1995)

COMPLEX STUDY OF THE PHYSIOLOGICAL ROLE OF CADMIUM II. EFFECT OF CADMIUM LOAD ON THE CADMIUM CONTENT OF EGGS

J. BOKORI[1], S. FEKETE[1], I. KÁDÁR[2] and M. ALBERT[3]

[1]Department of Animal Nutrition and [3]Department of Pathology, University of Veterinary Science, H–1400 Budapest, P.O. Box 2, Hungary; [2]Agrochemical and Plant Nutrition Department, Soil Research Institute of the Hungarian Academy of Sciences, Budapest, Hungary

(Received March 16, 1994)

The possibility of "cadmium (Cd) contamination" of eggs and the dynamics of Cd accumulation were studied. A total of 40 (4×10) Japanese quails weighing 155–200 g, being at the middle phase of egg production and kept on batteries in a climatized animal house were used. The birds were fed a standard quail layer diet and drank tap-water *ad libitum*. With the exception of the control group (Cd-0), the diet of the other three groups was supplemented with Cd sulphate homogeneously mixed in the diet so that it contained 75 mg (Cd-75), 150 mg (Cd-150) and 300 mg (Cd-300) per kg. During the 37-day feeding trial the quails' behaviour, health status and daily egg production were monitored and the birds were weighed weekly. The egg production of the experimental groups, particularly of those exposed to a high Cd load, rapidly decreased: at the highest Cd load (300 ppm) egg production completely ceased at days 10–11 of the trial. The total Cd content of the eggs rose already from the 3rd day of the feeding trial and by day 10 it reached a value of 0.777 mg/kg dry matter in the eggs of quails of group Cd-75. This was about five times the value measured in the control quails' eggs (0.165 mg/kg dry matter). The Cd content of the egg-white and egg-yolk (maximum values: 0.212 and 0.107 mg/kg dry matter) also increased to about 2 or 3 times that measured in the control eggs. The Cd content of eggs reached the peak in the 2nd week of the feeding trial, then started to decrease, and in the 3rd and 4th weeks not even the eggs laid by quails exposed to a high Cd load contained more Cd than about twice the concentration measured in the controls' eggs. The elucidation of this hitherto not studied process requires further investigations. The phenomenon may be due to a lack of the protein necessary for Cd transport and to the impairment of Cd absorption and of the excretory activity of the oviduct. The higher Cd concentrations measured in the egg were accompanied by markedly elevated Fe, K and S values. In the egg samples collected in the 4th week a substantial reduction in Ca and P concentration was observed. As regards the mineral elements measured in the egg-shell, a significant negative correlation was established between the concentration of Cd and those of Zn, Mg and Ca. Necropsy revealed severe

0236–6290/95/$ 4.00 © 1995 *Akadémiai Kiadó, Budapest*

emaciation, hepatic and renal degeneration. In many birds there was catarrhal enteritis, ovarian and oviductal atrophy. By light microscopic examination the follicular epithelial cells of the ovaries showed degeneration, atrophy of the glands in the tunica propria, tubulonephrosis in the kidney, regressive changes of exocrine glandular epithelial cells of the pancreas, and osteoclastic osteolysis with degeneration of osteoblasts in the tibiotarsal bone.

Key words: Cadmium load, cadmium content of eggs, egg production, Japanese quail, correlation, mineral elements, gross and histopathology

The quantity and quality of animal products depend, besides the genetic capabilities and health status of animals, primarily on the composition of the diet fed. The feeding of a diet fully conforming to the production purpose renders it possible to obtain good-quality products from healthy animals and, thus, permits a profitable production. If with its diet the animal permanently ingests substances harmful to the organism, this will first lead to a quantitative and qualitative impairment of production. Severe cases will directly result in animal health disturbances and may indirectly cause food hygienic problems. Such harmful substances include e.g. the environment-polluting microelements ingested with the feed, among them cadmium (Cd), an element which accumulates in feed constituents, in the animal organism and thus also in foodstuffs.

To date, only in few cases has the "Cd contamination" of foodstuffs reached a critical level. However, with the gradual accumulation of Cd in the environment the potential risks of that are ever higher. Precisely this is why such studies command increasing interest.

The present work addresses a topic forming part of the above subject, when it investigates the possibility of "Cd contamination" of eggs, an important poultry product which constitutes one of the major human food sources. The aim of the study was to conduct a model experiment in which Cd was mixed in the poultry diet and fed at different concentrations, to determine when and in what concentration Cd appeared in the egg and in its different constituents. A further objective was to monitor changes in egg production, egg quality and Cd content, as well as in the health status of layers.

Review of the literature

According to the available data of the literature, during the investigation of Cd metabolism and toxicity several authors have determined also the Cd content of eggs. However, only few researchers reported on studies aimed directly at egg Cd content.

According to certain authors (Krampitz et al., 1974) the dietary Cd concentration capable of eliciting physiological effects is between 3 and 61 mg, while others (Fox, 1983) put that limit at 5 μg (!!). This large difference is explained by the fact that the metabolism and adverse effects of Cd are greatly influenced by its interaction with other constituents of the diet, mainly with dietary Zn, Fe and Cu content (Fox et al., 1979; Bundscherer, 1984).

It is a generally held view that foods derived from animals kept under optimum conditions (except those prepared from the inner organs) usually contain only low amounts of Cd (Hapke, 1984; Weigert et al., 1984; Stolley, 1989). Besides the relatively low "Cd contamination" of foodstuffs, this is due to the very poor utilization rate of Cd, which has been demonstrated in experiments with labelled Cd (Fox et al., 1978). According to other authors (Stadelman and Prati, 1989), if large amounts of heavy metals (e.g. Cd, Cu, Pb) and Zn are present in the diet, these elements will appear in the eggs at a proportionally high concentration.

In order to study the physiological effects of Cd load as well as the accumulation and toxicity of Cd in the organs, Pritzl et al. (1974) conducted a Cd loading experiment on a total of 196 two-week-old Leghorn cockerels assigned to nine groups. They fed a diet containing 0, 400, 600, 700, 800 and 1,000 mg Cd ($CdCO_3$). The main findings of that study were the following: (*i*) the mass of inner organs (gizzard, gizzard mucosa, kidney) of cockerels fed a diet containing 700 ppm Cd had increased; (*ii*) deviations were observed in the colour, Cd and Zn concentration of the organs; mortality rose and feed conversion worsened in proportion to dietary Cd concentration; (*iii*) in their view, diets of more than 400 ppm Cd concentration should be considered highly toxic; (*iv*) according to the calculations based on their results, they defined the LD_{50} of Cd as 565 mg/kg of feed.

On the basis of extensive control tests, the Cd content of eggs, calculated for 1 kg of fresh (wet) substance, was reported to be 0.01 mg (Kaul et al., 1989) and 0.05 mg (Lindner, 1989).

Hardebeck et al. (1974) studied the accumulation of Cd in different parts of eggs by feeding a ^{115}Cd-labelled compound while Sülz (1973) after intravenous administration. In agreement with the findings of other authors (Hennig et al., 1971; Leach et al., 1979), they observed that Cd could be detected, mainly in the egg yolk, in very low amounts only. Sharma et al. (1979) fed a diet containing 0.3, 1.9 and 13.1 ppm Cd to pullets over a period of 6 months. After such Cd load they did not observe Cd accumulation in the bones and eggs. However, in the 6th month of the feeding experiment they detected Cd accumulation in the liver, kidneys and muscles, which was closely related to the dose of Cd and the duration of the Cd load.

In a 24-week feeding trial, Sülz et al. (1974) fed to layers diets containing 3 or 61 μg Cd. The feeding of the diet of higher Cd content led to a reduction in feed utilization and egg production. No changes were observed in egg-shell quality.

In a long-term (329-day) trial conducted with layers, Nesel and Voght (1977) fed a diet which contained 0.1, 5.1 and 10.1 μg Cd per g of diet. Such Cd supplementation of the diet caused no adverse effect on egg production or hatchability. Although Cd could not be detected in the egg-white, the egg-yolk of birds fed 5 and 10 μg Cd per g of diet contained 0.05 and 0.10 μg Cd per g, respectively.

Leach et al. (1979) studied the effects of Cd load in broiler chickens and laying hens fed diets of different Cd concentration (3, 12 and 48 μg Cd/g) for a relatively long period (6–12 weeks). The main results were the following: (*i*) all three levels of Cd load increased the Cd concentration of the kidneys, while only the two higher doses led to a rise in the Cd content of the liver and muscles; (*ii*) the Cd level of eggs was found to be very low, and a measurable rise in Cd level occurred only in the eggs of laying hens fed a diet containing 48 μg Cd per g; (*iii*) the feeding of 48 μg Cd per g of diet lowered the egg production and decreased egg-shell thickness.

Holcman and Smodis (1993) studied the concentration of four elements (As, Cd, Hg and Pb) in the eggs laid by layers kept under loose conditions or on batteries. They established that the largest amount of As, Cd and Hg accumulated in the egg-white of lyophilized egg samples. In the same egg samples, more Pb was present in the egg-yolk. In the yolk of fresh eggs, Cd, As and Pb occurred in larger amounts but Hg did not. The average concentration of the four metals was higher in the eggs of hens kept loose than in those of hens kept under large-scale conditions.

Materials and methods

In order to monitor Cd accumulation, an experiment was conducted using a total of 40 Japanese quails (four groups of 10 birds each) being at the peak of egg production and weighing 155–200 g. The quails were housed in a climatized animal house on two tiers of a battery, at approximately the same height. In the first week of the experiment all birds received a standard quail layer diet (Table 1). Subsequently, with the exception of the control group (Cd-0), the diet fed to the other three groups was supplemented with Cd ($CdSO_4$) to contain 75 (Cd-75), 150 (Cd-150) and 300 (Cd-300) mg/kg of diet, respectively. The feeding trial lasted from 16 June to 22 July (for a total of 37 days). Feed and drinking water were available *ad libitum*.

The quails' behaviour, health status and feed consumption were checked daily throughout the trial. The egg production and egg quality were monitored in all four groups. Eggs weighing 8–10 g and laid within a 24-hour period were collected in each group, washed with running tap-water and then with distilled water, wiped dry with a paper swab and placed into sealable plastics containers. The lat-

ter were stored in a refrigerator at 4 °C until the end of the feeding trial or until used for Cd assay. At that time, separate samples were prepared from the eggs for the determination of mineral element content. The 20 samples represented eggs from all groups and all parts of the eggs (whole egg, egg-white, egg-yolk, egg-shell). If sufficiently large numbers of eggs were available, the date of egg-laying was also taken into consideration to monitor the dynamism of Cd accumulation. The samples prepared from the mixture of one or more eggs were carefully dried to constant weight in a drying oven at 100 °C. The suitably pretreated samples were assayed for 22 elements using a JY24 type Joben Yvon ICP instrument of sequential mode of operation.

Table 1

Composition of the quail layer diet

	kg
Maize	40.00
Wheat	16.30
Extr. soybean 48%	20.00
Extr. sunflower groats (40.0%)	5.30
Meat meal (54%)	3.00
Energomix-40 (*)	6.00
AP-17	2.90
Fodder lime	5.70
Feed salt	0.30
Standard premix	0.50
Total	100.00

(*) Guaranted content of Energomix-40:

Dry matter	%	90.00
Metabolizable energy	MJ/kg	21.70
Crude protein	%	4.00
Crude fat	%	40.00
Crude fibre	%	2.00

The number and body mass of animals that died or were culled during the feeding trial are shown in Table 2. The carcases were weighed, then subjected to detailed gross pathological while their organs to microbiological examination.

On day 42 of the feeding trial, 8 experimental quails (3 birds each from group Cd-75 and group Cd-150 and 2 birds from group Cd-300) and 3 control birds (Cd-0) were killed by bleeding and subjected to detailed gross and histopathological examination.

Table 2

Losses during the feeding trial

Day of feeding trial	Group	Number of deaths	Body mass (g)
2	Cd-0	1	180
3	Cd-75	1	190
37	Cd-75	1	138
37	Cd-75	1	100
23	Cd-150	1	130
24	Cd-150	1	120
30	Cd-150	1	145
36	Cd-150	1	138
37	Cd-150	1	100
4	Cd-300	1	110
14	Cd-300	1	70
27	Cd-300	1	135
30	Cd-300	1	118
31	Cd-300	1	145
31	Cd-300	1	140
Total	–	15	–

At necropsy, samples were taken from nine organs (liver, pancreas, kidney, heart and skeletal muscle, ovary, magnum and uterus of the oviduct, and the proximal end of the tibiotarsal bone). The samples were fixed in 8% neutral formaldehyde solution, embedded in paraffin, and sectioned as described earlier (Bokori et al., 1993). The sections were stained with haemalum and eosin. The bones were embedded after decalcification with Ca-EDTA.

Results

The egg production of quails and the quality of eggs are shown in Table 3 and Fig. 1, the number and body mass of birds that died are presented in Table 2, while the concentrations of the 15 elements measured in the whole eggs, egg-white, egg-yolk and egg-shell are summarized in Table 4. The accumulation of Cd in the whole eggs is illustrated in Fig. 2. The correlations calculated among 13 mineral elements measured in the egg-shell are presented in Table 5.

At necropsy, slight (Cd-75) or severe (Cd-300) emaciation was observed as compared to the control. The ovaries of the control birds contained numerous maturing and mature follicles, while in the oviduct an egg of calcic shell was found. In the ovaries of the treated quails the number of live follicles was reduced and the larger follicles showed degeneration. The oviduct of only one of these birds contained an egg of calcic shell. The most expressed ovarian and oviductal atrophy was found in group Cd-300. In quails of groups Cd-150 and Cd-300 the tubular bones could easily be cut through with a knife as a result of decalcification.

Light microscopic examination. As compared to the control, the follicular epithelial cells of the *ovaries* showed degeneration. The ovary of quails of group Cd-300 contained some large degenerated follicles and the primary follicles also showed signs of degeneration (Fig. 3).

In the magnum portion of the treated birds' *oviduct,* the glandular epithelial cells of the tunica propria became deficient in eosinophilic granules (protein granules) and their cytoplasm became vacuolated. The glands became atrophic and contained cyst-like dilatations in some places. The tunica propria of the uterus showed oedema and glandular atrophy (Fig. 5). The histological picture of the intact uterus of control quails is shown in Fig. 4.

In the treated quails' *kidney*, tubulonephrosis accompanied by regeneration of the tubular epithelial cells was observed. Here and there the nuclei of the regenerating cells contained inclusion-like eosinophilic bodies.

Already in quails exposed to 75 ppm Cd, the cytoplasm of exocrine glandular epithelial cells of the *pancreas* showed a depletion of eosinophilic granulation in the apical part of the cell. The nuclei of some cells exhibited regressive changes (karyopyknosis, karyorrhexis). The cytoplasm of the cells contained so-called hyaline bodies. Several acini contained degenerated cells that had been expulsed from the cell row into the lumen of the gland.

The *myocardium* of the treated animals showed perinuclear oedema of the myocardial cells and blurred striation of the myocardial fibres. In circumscribed areas, the myocardial cells showed eosinophilic staining (focal necrosis). The skeletal muscle contained no major light microscopic changes.

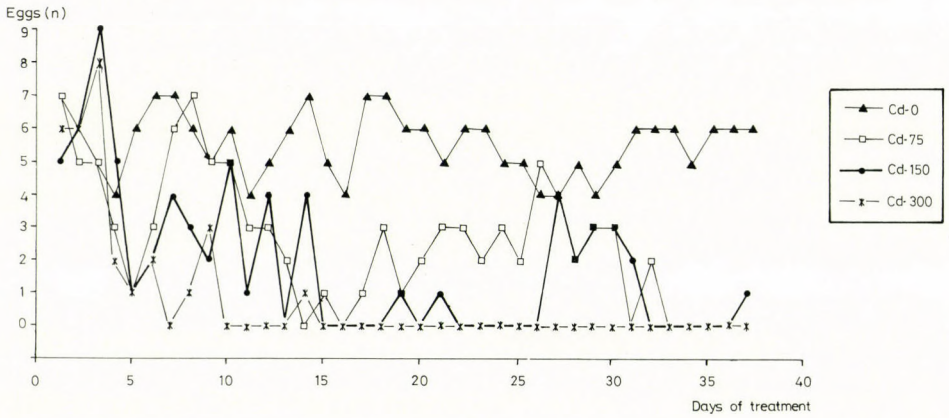


Fig. 1. Number of eggs produced by different groups of quails during the cadmium load

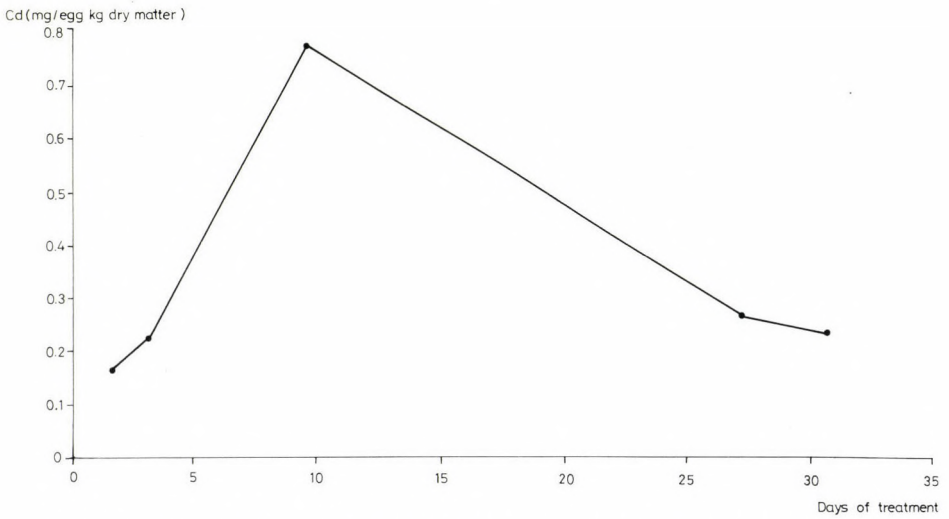


Fig. 2. Changes in the cadmium concentration of the whole egg over time, under 75 ppm cadmium load

Table 3

Changes in egg production and egg-shell quality in the control group (Cd-0) and in the three cadmium-exposed groups (Cd-75, Cd-150, Cd-300)

Days of the feeding trial	Number of birds (n) and designation of groups											
	n	Cd-0		n	Cd-75		n	Cd-150		n	Cd-300	
1	10	7		10	7	(1)	10	5	(1)	10	6	(1)
2	9	6		10	5	(2)	10	6	(2)	10	6	(1)
3	9	5		9	5		10	9		10	8	(3)
4	9	4		9	3		10	5	(1)	9	2	(1)
5	9	6	(1)	9	1		10	1		9	1	
6	9	7		9	3		10	2		9	2	
7	9	7		9	6	(2)	10	4	(1)	9	0	
8	9	6		9	7	(2)	10	3		9	1	
9	9	5		9	5		10	2		9	3	(2)
10	9	6		9	5		10	5	(2)	9	0	
11	9	4		9	3		10	1		9	0	
12	9	5	(1)	9	3		10	4		9	0	
13	9	6		9	2		10	0		99	0	
14	9	7		9	0		10	4		8	1	(1)
15	9	5		9	1		10	0		8	0	
16	9	4		9	0		10	0		8	0	
17	9	7		9	1		10	0		8	0	
18	9	7		9	3	(1)	10	0		8	0	
19	9	6	(1)	9	1		10	1	(1)	8	0	
20	9	6		9	2	(1)	10	0		8	0	
21	9	5		9	3		10	1		8	0	
22	9	6		9	3	(1)	10	0		8	0	
23	9	6	(1)	9	2		9	0		8	0	
24	9	5		9	3		8	0		8	0	
25	9	5		9	2		8	0		8	0	
26	9	4		9	5		8	0		8	0	
27	9	4		9	4		8	4		7	0	
28	9	5		9	2		8	2		7	0	
29	9	4		9	3	(1)	8	3		7	0	
30	9	5		9	3		7	3	(1)	6	0	
31	9	6	(1)	9	0		7	2		4	0	
32	9	6		9	2		7	0		4	0	
33	9	6		9	0		7	0		4	0	
34	9	5		9	0		7	0		4	0	
35	9	6		9	0		7	0		4	0	
36	9	6		9	0		6	0		4	0	
37	9	6		9	0		5	1	(1)	4	0	
Total:		206	(5)		95	(11)		68	(10)		30	(9)

*Numbers in parentheses indicate the soft-shelled and broken eggs

Table 4

Mineral element content of the egg samples

Egg samples tested			Elements (mg/kg dry matter)														
Origin	Day of collection	Type of sample (n)	Cd	Fe	Zn	Cu	Mn	Mo	Sr	Ba	B	Ca	P	Mg	Na	K	S
Cd-0	1–2	Whole egg (10)	0.165	53.1	36.3	5.31	0.428	0.44	83.8	3.9	1.31	96633	7080	2084	5070	6695	5191
Cd-75	3	Whole egg (5)	0.226	44.3	30.1	2.86	0.55	0.196	130.0	5.65	1.53	75477	6167	2051	5461	7616	6472
Cd-75	9	Whole egg (5)	0.777	40.4	28.9	2.7	0.478	0.194	160.0	6.89	0.37	84385	6443	2215	4992	7214	5873
Cd-75	27	Whole egg (4)	0.270	32	18.8	2.78	0.131	0.174	162.0	4.26	0.40	84532	5247	2554	5226	7837	6494
Cd-75	30–31	Whole egg (3)	0.236	22.2	17.1	2.89	0.158	–	128.0	5.25		89312	5667	2412	6143	7820	6500
Cd-150	3–4	Whole egg (13)	0.195	49.5	36.9	3.21	0.519	0.32	138.0	6.07	1.85	91835	5990	1903	4757	7810	5461
Cd-300	2–3	Whole egg (10)	0.282	61.9	38.2	2.65	0.493	0.36	118.0	8.39	1.89	85740	6389	2160	4247	5951	4897
Cd-300	4–9	Whole egg (4)	0.250	63.5	38.7	2.82	0.281	0.32	114.0	6.05		82495	6278	1533	5119	8057	5228
Cd-0	3–5	Egg-white (10)	0.078	5.88	0.07	3.34	–	0.641	1.2	0.6	1.78	260	1151	712	23221	21287	14460
Cd-75	28–30	Egg-white (8)	0.192	7.73	0.48	2.87	–	0.407	1.5	0.9	0.54	926	1254	821	16456	16580	14130
Cd-75	32–37	Egg-white (3)	0.212	9.6	7.88	3.55	0.48	0.596	2.5	0.73	0.50	872	2296	459	12089	12500	10940
Cd-150	9–12	Egg-white (10)	0.119	7.22	1.93	4.82	0.47	0.234	1.7	0.8	0.61	390	1161	673	17314	21272	14340
Cd-0	6–7	Egg-yolk (10)	0.060	86.5	56.5	3.39	0.81	0.62	2.4	0.87	1.80	2889	9051	283	1839	2783	3991
Cd-75	28–30	Egg-yolk (8)	0.383	91.7	40.8	2.62	0.72	0.43	4.1	1.63	1.41	3072	10982	320	1728	2915	3852
Cd-75	32–37	Egg-yolk (3)	0.268	79.1	58	2.68	1.15	0.5	5.5	1.41	–	2779	9775	386	3090	3352	4582
Cd-150	34–37	Egg-yolk (10)	0.187	91.2	63.2	3.38	1.22	0.26	6.4	2.29	–	2902	8086	338	2937	4660	4352
Cd-0	6–7	Calcic egg-shell (10)	0.045	1.9	0.77	3.05	0.05	0.48	2.7	8.4	–	305689	2670	6153	4827	4819	3805
Cd-75	4–10	Calcic egg-shell (8)	0.048	1.4	0.91	2.55	0.04	0.23	4.2	13.9	–	289405	3203	6303	4714	4889	5461
Cd-75	38–31	Calcic egg-shell (3)	0.105	1.4	8.99	2.78	–	–	2.9	11.7	–	240367	1934	5048	4628	6288	4396
Cd-150	32–37	Calcic egg-shell (3)	0.107	1.7	8.91	3.36	0.06	–	3.3	12.7	–	260734	3280	5709	4707	6791	4701

Table 5

Correlations among mineral elements present in the egg-shell under cadmium load

	Cd	Fe	Zn	Cu	Sr	Ba	Ca	P	Mg	Na	K	S
Cd	1.000											
Fe	0.693	1.000										
Zn	–0.999	0.672	1.000									
Cu	0.441	–0.027	0.434	1.000								
Sr	–0.265	0.509	–0.294	–0.507	1.000							
Ba	–0.623	–0.981	–0.605	0.211	–0.556	1.000						
Ca	–0.931	–0.741	–0.936	–0.089	0.143	0.741	1.000					
P	–0.273	0.189	–0.308	0.258	0.633	–0.089	0.458	1.000				
Mg	–0.855	–0.425	–0.873	–0.127	0.482	0.437	0.923	0.729	1.000			
Na	–0.736	–0.834	–0.736	0.272	–0.201	0.890	0.917	0.374	0.760	1.000		
K	0.983	0.702	0.977	0.560	–0.220	–0.603	–0.858	–0.107	–0.750	–0.645	1.000	
S	–0.031	0.698	–0.060	–0.482	0.969	–0.744	–0.106	0.525	0.255	–0.430	–0.004	1.000



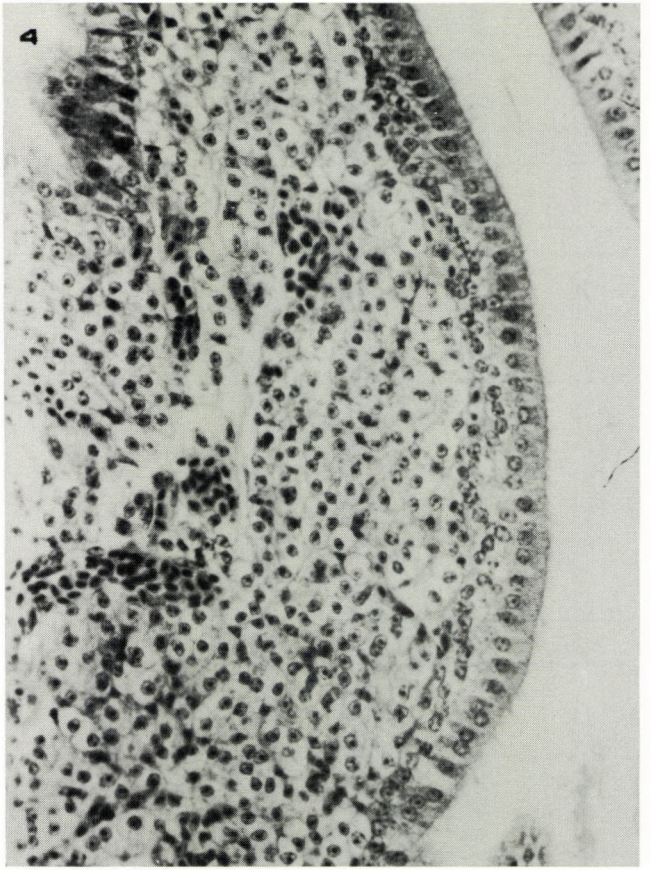


Fig. 3. Histological picture of the ovary from a laying quail fed 300 ppm Cd for 6 weeks. Note atrophy of the ovary. H.–E., × 250
Fig. 4. Histological picture of the intact uterus from a control laying quail. H.–E., × 50

The *liver* of the Cd-treated quails exhibited pronounced proliferation of the biliary ductules. At the marginal parts of the lobules the hepatocytes showed cholestasis. The nuclei of several hepatocytes contained eosinophilic inclusion-like formations (nuclear degeneration). Numerous degenerated hepatocytes that had left the cell row could be seen in Disse's spaces.

The *tibiotarsal bone* of the control quails showed osteothesaurismosis, a condition typically observed in the laying season (Fig. 6). With elevation of the Cd dose, the treated birds showed a depletion of bone tissue (Fig. 7). The bone trabeculae had become thin and the number of osteoclasts increased (osteoclastic osteolysis). The osteoblasts showed signs of degeneration (vacuolation of the cytoplasm, karyopyknosis, complete cell necrosis). In group Cd-300, proliferating osteoblasts could be seen on the thin bone trabeculae; however, osteoid production could not be observed yet (Fig. 8).

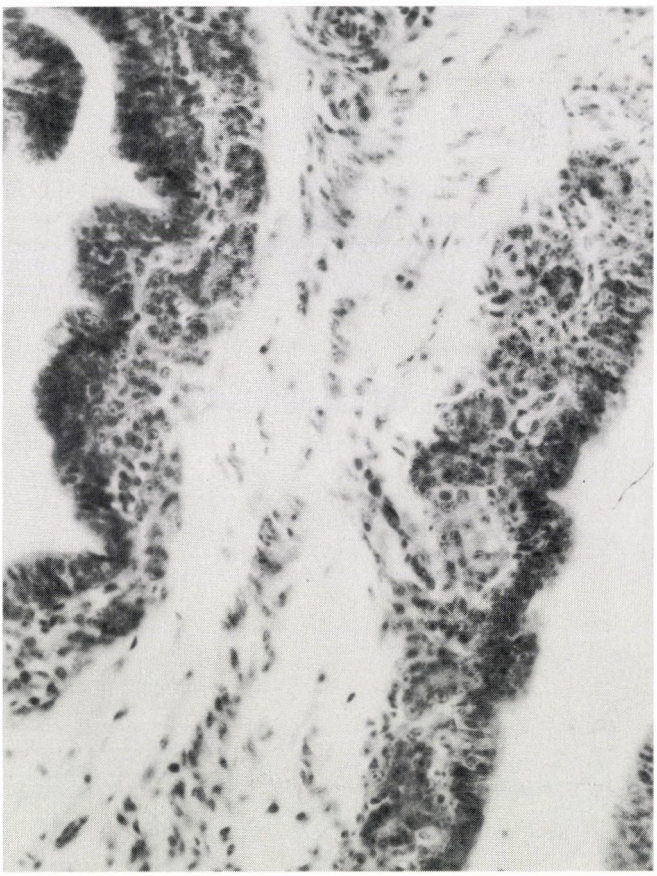


Fig. 5. Histological picture of the uterus from a laying quail fed 300 ppm Cd for 6 weeks. Note severe atrophy of the glands in the tunica propria. H.–E., × 250

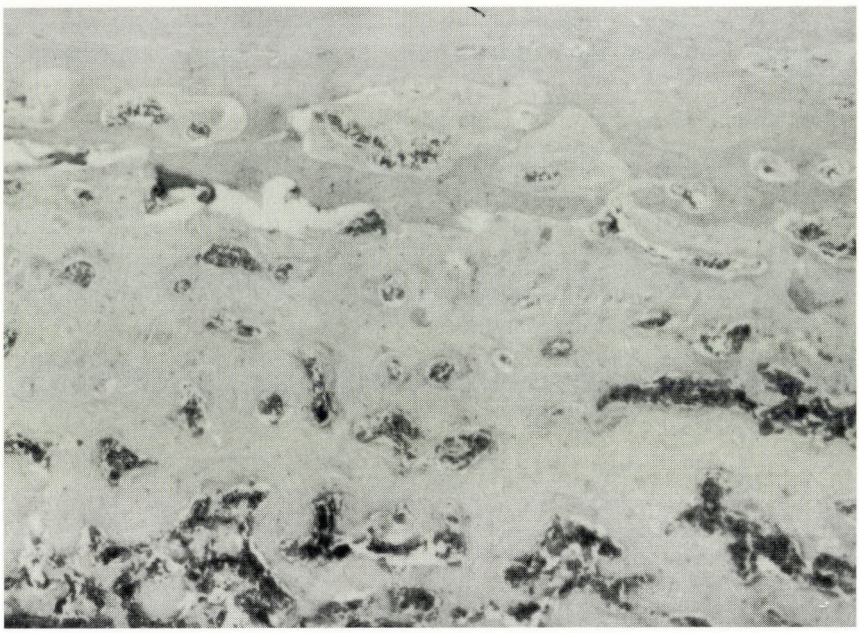


Fig. 6. Histological picture of the tibiotarsal bone from a control laying quail. Osteothesaurismosis. H.–E., × 50

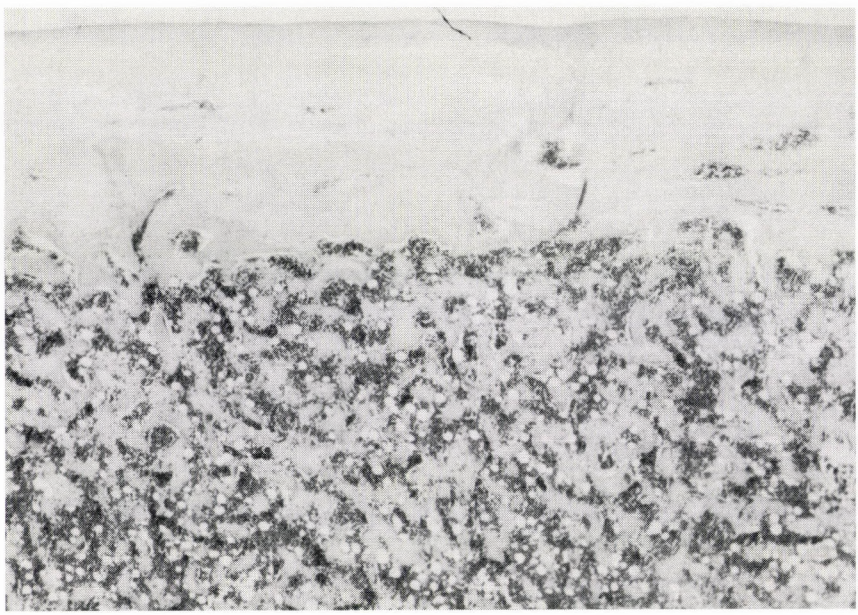


Fig. 7. Histological picture of the tibiotarsal bone from a laying quail fed 150 ppm for 6 weeks. Note substantial depletion of the bone tissue. H.–E., × 50

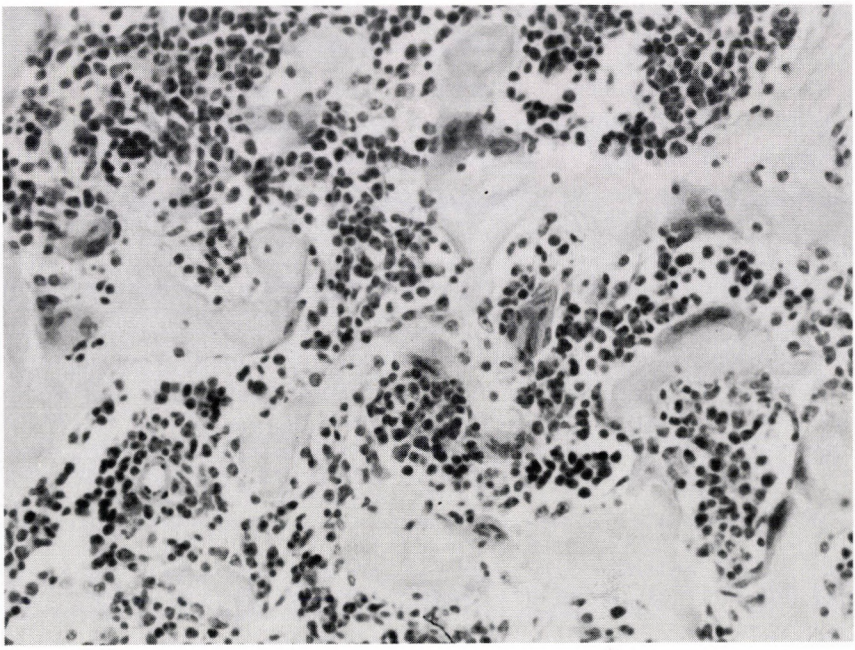


Fig. 8. Histological picture of the tibiotarsal bone from a laying quail fed 75 ppm Cd for 6 weeks. Osteoclastic osteolysis. H.–E., × 250

Discussion

In the week preceding the start of the feeding trial, all groups laid 5–7 eggs daily, ate well and showed no pathological signs. In the first 10 days of feeding the Cd-containing diet, after a 1- to 2-day transient increase egg production started to decrease slowly and less markedly in the groups fed 75 and 150 mg Cd, while in the group fed 300 mg Cd it underwent a striking drop already from day 4: after day 5 that group laid only 1–3 eggs per day or no eggs at all, while from days 10–11 it completely ceased to lay eggs. During the 37-day feeding trial total egg production decreased at an inverse ratio to dietary Cd content and was 95, 68 and 30 eggs in the Cd-75, Cd-150 and Cd-300 groups, respectively. In addition, the number of soft-shelled eggs and eggs with cracked shells also rose (Table 3). These results are consistent with the observations of Leach et al. (1979) and Sülz et al. (1974). The birds fed a diet of higher Cd content gradually lost their appetite, emaciated and many of them developed diarrhoea. One control and 14 experimental quails (a total of 15 birds) died or were lost during the feeding trial (Table 2). Necropsy revealed severe emaciation, hepatic and renal degeneration,

catarrhal enteritis and, in many birds, oedematous infiltration of the subcutaneous and perirenal connective tissue.

Based upon the mineral element content of the egg samples (Table 4), the following main conclusions can be drawn. (*i*) The total Cd content of *eggs* increased already from day 3 of the feeding trial and e.g. in the eggs of quails fed 75 mg Cd per kg diet its maximum (0.777 mg/kg dry matter) reached five times that of the controls (0.165 mg/kg dry matter). Subsequently, Cd concentration of the eggs gradually decreased over time, and at the end of the feeding trial it was only 0.236 mg/kg dry matter. The Cd content of eggs laid by birds fed 150 and 300 mg Cd per kg of diet was not higher: moreover, in these groups the maximum Cd concentration was only 0.282 mg/kg dry matter. Taking into consideration the dry matter content of eggs (approx. 35%), the average Cd values measured in the eggs of control birds came close to the values reported by Kaul et al. (1989) and Lindner (1989), and confirm that Cd passes into the egg. (*ii*) The Cd content of the *egg-white* nearly trebled: it increased from the average value of 0.078 mg/kg dry matter to 0.212 mg/kg dry matter. As opposed to observations made by others (Hennig et al., 1971; Sülz, 1973; Hardebeck et al., 1974), a slightly higher Cd concentration was measured in the *egg-yolk.* (*iii*) The Cd concentration of the *egg-shell* underwent a less rapid and max. twofold increase (in the 2nd and 3rd weeks, from 0.045 mg to 0.107 mg/kg dry matter).

In summary, it can be established that, as a result of feeding Cd-containing diets, the total Cd content of eggs underwent a substantial and maximum 5-fold increase already from day 3 of feeding. As compared to the control, the Cd content doubled or trebled in the egg-white, egg-yolk and somewhat later also in the egg-shell. The rapidly appearing "Cd contamination" reached the peak in the 2nd week after the start of feeding the Cd-containing diet, then it decreased and in the 3rd and 4th weeks it did not exceed the control value even in birds exposed to a high Cd load. This may be due to a reduction in the transport of Cd bound to a protein produced by the liver, to an impairment of Cd absorption or of the Cd-excreting activity of the oviduct. Elucidation of this question, which has not yet been studied by any authors, requires further experiments.

The following interactions among mineral elements in the egg have been demonstrated in this study. (*i*) The rise in egg Cd content decreased the Zn, Fe, Cu, Mo, B, Ca and mainly the P concentration of the egg, while it left unchanged or increased the concentration of the other elements. (*ii*) Parallel to Cd accumulation, the S, Ba and Mg content of the eggs rose while their Fe and Cu content tended to decrease. (*iii*) The highest Cd concentration was accompanied by elevated Fe, Zn, Sr, Ca and P values in the egg-white and higher Fe, Sr, Ba, Ca and P levels in the egg-yolk. At the same time, the Cu and Mo concentration of the egg-yolk was lower. (*iv*) Elevated Cd content of the egg-shell was accompanied by markedly increased Fe, K and S values, while in samples taken in the 4th week a markedly lowered Ca and P content was found. The observed behaviour of Ca, P

and Zn supports the statement of several authors (Jacobs et al., 1974; Fox et al., 1979; Bundscherer, 1984) that these elements are in a negative interaction with Cd metabolism. A similar observation was reported by Leach et al. (1979) regarding the total Zn content of eggs.

Of the 8 elements not shown in Table 4, As, Hg, Se, Co and Al could not be detected in any of the samples. Cr was detected in the egg-shell of one egg (0.532 mg/kg dry matter), Pb in 9 egg samples (0.079–1.67 mg/kg dry matter), and Ni in 5 egg samples (0.212–0.615 mg/kg dry matter).

Of the mineral elements measured in the egg-shell, calculations revealed a significant negative correlation among Zn, Ca, Mg and Cd (Table 5).

Acknowledgement

This work was supported by the National Research Fund (OTKA), project no. 1270.

References

Bokori, J., Fekete, S., Kádár, I., Vetési, F. and Albert, M. (1993): Complex study of the physiological role of aluminium II. Aluminium tolerance tests in broiler chickens. Acta Vet. Hung. **41,** 235–264.

Bundscherer, B. (1984): Der Einfluss der Zn-Zufuhr und verschiedener Bindungsformen des Cadmiums auf die Cadmium-Retention in Leber und Nieren beim Hühnerküken. Vet. Med. Diss., München, 1984.

Fox, M. R. S. (1983): Fed. Proc; Fed. Am. Soc. Exp. Biol. **42,** 1726. In: Mertz, W. (ed.): Trace Elements in Human and Animal Nutrition. 5th edition. Academic Press, New York, 1986.

Fox, M. R. S., Jacobs, M. R., Jones, L. O. A., Fry, B. E. and Hamilton, R. P. (1978): In: Kirchgessner, M. (ed.) Trace Element Metabolism in Man and Animals — 3. Univ. Münich, F. R. G. p. 327.

Fox, M. R. S., Jacobs, M. R., Jones, L. O. A. and Fry, E. B. (1979): Effects of nutritional factors on metabolism of dietary cadmium at level similar to those of man. Environm. Health Perspect. **28,** 107–114.

Hapke, H. J. (1984): Metallbelastungen von Futter-und Lebensmittel, Akkumulationen in der Nahrungskette. In: Merian, E. (ed.) Metalle in der Umwelt Verlag Chemie, Weinheim, 1984.

Hardebeck, H., Sülz, M. and Krampitz, G. (1974): Die Wirkung von Cadmium in Organismus. Arch. Geflügelk. **3,** 100–103.

Hennig, A., Georgi, K. and Jeroch, H. (1971): Zur kontraceptiven Wirkung des Cadmiums. Arch. Exp. Veterinärmedizin **25,** 793–797.

Holcman, A. and Smodis, B. (1993): Heavy metal content in hen's eggs. In: Anke, M., Meissner, D. and Mills, F. C. (eds) Trace Elements in Man and Animals — TEMA 8. Verl. Media Touristik, Jena, 1993. p. 249.

Jacobs, R. M., Fox, M. R. S., Lee, O. A. and Harland, F. B. (1974): Increased duodenal and decreased hepatic and renal Cd in Japanese quail fed supplements of Zn, Mn and Cu. Fed. Proc. **33,** 668.

Kaul, A., Lingk, W., Siewert, E., Somogyi, A., Weigert, P. and Wirth, E. (1989): Schwermetallgehalte in Lebensmitteln tierischen Ursprung des deutschen Marktes. In: Grossklaus, E. (ed.) Rückstande von Tieren stammenden Lebensmitteln. Paul Parey Verlag, Berlin, pp. 127–135.

Krampitz, G., Sülz, M. and Hardebeck, H. (1974): Die Wirkung von Cadmiumgaben auf das Huhn. Arch. Geflügelk. **3,** 86–90.

Leach, R. M., Wei-Li Wang, K. and Baker, E. D. (1979): Cadmium and the food chain: The effect of dietary cadmium on tissue composition in chicks and laying hens. J. Nutr. **109,** 437–443.

Lindner, J. (1989): Untersuchungen über endogene und exogene Einflüss auf die Cadmium-, Blei-, Quecksilber-, Kupfer- und Zinkgehalte in Organen und Muskulatur von Feldhasen. Thesis, Giessen, 1989.

Nezel, F. and Voght, H. (1977): The effect of various levels of cadmium in broiler and laying rations on the performance of the birds and on residues in tissues and eggs. Arch. Geflügelk. **41,** 81–86.

Pritzl, C. M., Lie, H. Y., Kienholz, W. E. and Whiteman, E. C. (1974): The effect of dietary cadmium on development of young chickens. Poultry Sci. **53,** 2026–2029.

Sharma, P. R., Street, C. J., Verma, P. M. and Shupe, L. J. (1979): Cadmium uptake from feed and its distribution to food products of livestocks. Environm. Health Perspect. **28,** 59–66.

Stadelman, J. W. and Prati, E. D. (1989): World's Poultry Science Journal **45,** 247–266. In: Anke, M., Meissner, D. and Mills, F. C. (eds) Trace Elements in Man and Animals — TEMA 8. Verlag Media Touristik, Jena, 1993.

Stolley, U. (1989) Der Einfluss verschiedener Vitamine und Spurenelemente auf die Cadmiumretention in der Niere beim Hühnerküken. Thesis, München, 1989.

Sülz, M. (1973): Die Wirkung von Cadmiumgaben auf das Huhn. Thesis, Bonn, 1973.

Sülz, M., Hardebeck, H. and Krampitz, G. (1974): Long-term influence of cadmium on feed consumption, weight gain, egg performance and eggshell qality of laying hens. Arch. Geflügelk. **38,** 150–154.

Weigert, P., Müller, J., Klein, H., Zufelde, P. K. and Hillebrand, J. (1984): Arsen, Blei, Cadmium und Quecksilber in und auf Lebensmitteln. ZEBS — Hefte, 1. 31 und 6. 7. 1984.

Acta Veterinaria Hungarica 43 (1), pp. 63–77 (1995)

IN VITRO AND *IN VIVO* MOTILITY STUDIES OF ^{99m}Tc HM-PAO LABELLED SPERM CELLS

L. BALOGH[1], F. SZÁSZ[2], L. ZÖLDÁG[2], Gy. HUSZENICZA[2], L. TÓTH[3], Gabriella DABASI[4] and Gy. JÁNOKI[1]

[1]Department of Applied Isotopes, National Frédéric Joliot-Curie Research Institute for Radiobiology and Radiohygiene, H–1221 Budapest, Anna u. 5–7, Hungary; [2]Department and Clinic of Obstetrics and Reproduction, University of Veterinary Science, H–1400 Budapest, P.O. Box 2, Hungary; [3]Isotope Diagnostics Laboratory, Ferenc Jáhn Hospital, H–1204 Budapest, Köves út 2–4, Hungary; [4]Clinic of Radiology, Semmelweis University Medical School, H–1082 Budapest, Üllői út 16, Hungary

(Received February 16, 1994)

The motility of ^{99m}Tc HM-PAO radiolabelled sperm cells, labelled as described previously (Balogh et al., 1992), was studied. The active migration of spermatozoa was demonstrated in capillary tubes containing bovine oestrous mucus, using an *in vitro* motility analyzer. Like nonlabelled sperm cells, the labelled spermatozoa covered a 4–5 cm distance in the capillary tubes during a 10-min run. *In vitro* motility testing of labelled spermatozoa from different animal species (cattle, rabbit, sheep, horse) did not reveal significant differences. The distribution of spermatozoa within the female genital tract was studied in a previously described animal model (rabbit) and in two new models (sheep, chicken). This method enables the determination and visualization of sperm distribution by a noninvasive technique. The results of *in vivo* motility tests pave the way for the introduction of a method of unprecedented specificity, which serves for studying the penetrability of the oviduct to spermatozoa.

Key words: ^{99m}Tc HM-PAO labelled sperm cells, *in vitro* and *in vivo* motility studies, new animal models, noninvasive scintigraphic technique, veterinary nuclear medicine

Motility is a very important property of male germ cells of different species, and is closely correlated with fertilizing capacity (Kummerfeld et al., 1981). The description and quantitation of sperm motility require complex and expensive tests. The simplest and least accurate method is to study the motility of sperm cells on a heated slide under a stereomicroscope; however, this procedure is inaccurate and subjective even if performed by a highly experienced specialist. *In vitro* instrumental tests, ranging from runs in a simple capillary tube to computer-controlled analyzers demanding a high level of instrumentation, provide much more information independent of the examiner (Suttiyotin and Thwaites, 1992; Young et al., 1992). Truly "life-like" data, however, can be obtained only from *in vivo*

0236–6290/95/$ 4.00 © 1995 *Akadémiai Kiadó, Budapest*

transport studies. In that case, the monitoring of sperm migration represents the biggest difficulty, as until quite recently only invasive techniques were available for determining the distribution of sperm within the female genital tract (Templeton and Mortimer, 1980; Overstreet and Tom, 1982; Smith et al., 1987).

There have been many efforts to label spermatozoa with radioisotopes. The results of the first attempts were not reassuring because of the poor labelling efficiency of ^{125}I and ^{131}I isotopes (Oliphant et al., 1977; Lorton et al., 1980) and the vigorous motility-inhibiting effect of ^{67}Ga and ^{111}In isotopes (Lorton et al., 1980; Olive et al., 1987). Experiments with $^{99m}TcO_4^-$ and ^{99m}Tc HM-PAO (hexamethylpropylene amine oxime) yielded promising results (Lorton et al., 1980; Bockisch et al., 1989; Bockisch, 1990; Bockisch, 1993).

The ^{99m}Tc HM-PAO used in this work is currently one of the radiopharmacons most extensively used in nuclear medicine. For several years, it has been known in clinical practice and research as a compound suitable for labelling leucocytes and platelets (Danpure et al., 1986; Peters et al., 1986; Becker et al., 1987; Becker and Börner, 1988; Danpure and Osman, 1988; Becker et al., 1989; Papós et al., 1989). Besides labelling blood cells, it is widely used for scintigraphic examination of the brain (Biersack et al., 1987; Podreka et al., 1987; Langen et al., 1989). As regards its mechanism of action, it is supposed that the initially lipophilic molecule can cross the cell membrane, then — following structural isomeric transformation in the plasma — the compound assumes a hydrophilic character and, thus, is no longer capable of penetrating the membrane. The ^{99m}Tc HM-PAO "trapped" in this manner, acting as a chemical microsphere, is an aspecific labelling agent of cells (Kung and Blau, 1980).

The aim of the experiments presented in this paper was to develop a method suitable for studying the *in vitro* migration of spermatozoa in a capillary tube, and to attempt the monitoring of sperm cell motion in live female animals by a noninvasive technique. Spermatozoa labelled with ^{99m}Tc HM-PAO as described by us earlier (Balogh et al., 1992) were used. Radiolabelled spermatozoa are required to show the following properties: stable labelling of high efficiency, easily detectable gamma radiation, and maintenance of the highest possible viability and motility. Based upon data of the literature and our own results, spermatozoa labelled with ^{99m}Tc HM-PAO are suitable for *in vitro* and *in vivo* motility tests.

Materials and methods

Semen collection and separation

Samples from fresh ejaculates (of rabbits, horses and bulls) and deep-frozen semen (of bulls) were used. Fresh semen samples were collected with an artificial vagina. Deep-frozen bovine semen samples were derived from the same Red

Lincoln breeding bull of the National Artificial Insemination Station (Gödöllő). After semen collection or the thawing of deep-frozen semen, the samples were stored in a water-bath of 37 °C. Before use, a cell count was made (in Buerker chamber), mass motility was assessed by examination in a stereomicroscope, and the ratio of viable and dead spermatozoa was determined by eosin-nigrosin staining. The spermatozoa were separated from the plasm and from the diluent by centrifugation at 600 *g* for 5 min.

Collection of oestrous mucus from cows

Bovine cervical mucus was collected from oestrous cows by rectal massaging of the vagina (Kummerfeld et al., 1981). The transparent, mucinous, contamination-free oestrous mucus was collected in a sterile glass, then stored in liquid nitrogen (–192 °C) in a 5-ml capillary tube (Minitüb GmbH, Germany) until used.

Labelling of cells

The LEUCO-SCINT kit developed for human therapeutic purposes at the National Frédéric Joliot-Curie Research Institute for Radiobiology and Radiohygiene was used in this study. The kit contains 0.18 mg HM-PAO, 0.00228 mg $SnCl_2$, and 1.51 mg sodium pyrophosphate. The $^{99m}TcO_4^-$ needed for labelling was obtained from a Drygen (Sorin Biomedica) generator. Incubation with $^{99m}TcO_4^-$ solutions of different activity (80–1,000 MBq) was done in 0.4–0.6 ml physiological saline for 10 min. The lipid-soluble component of the ^{99m}Tc HM-PAO solution thus prepared was determined according to Ballinger et al. (1982) after incubation at room temperature for 5 and 60 min.

The sperm cell suspension separated in the above manner was carefully resuspended in 0.4–0.6 ml of the labelled ^{99m}Tc HM-PAO solution immediately after separation, then was incubated in a water-bath of 37 °C for 10 min. Subsequently the incubating fluid was separated from the cellular phase by centrifugation at 600 *g* for 5 min as described earlier, washed in physiological saline, and centrifuged again. Then the cell sediment was resuspended in the original plasma of fresh semen or in the diluent of deep-frozen bull semen (Laiciphos, I.M.V. Cassou, France). Labelling efficiency was determined from the activity ratio of the cell suspension and the two supernatants. The survival rate of spermatozoa was assessed after eosin-nigrosin staining while their motility by the estimation of mass motility. In one case, rabbit spermatozoa were labelled with ^{111}In-tropolone according to the method described for human platelets (Dewanje et al., 1984).

In vitro motility studies

The heatable motility analyzer tilt at an angle of 70°, currently under development at the Pannon University of Agricultural Sciences, Kaposvár, was used in this study. Spermatozoa labelled with ^{99m}Tc HM-PAO were stored in a plastics container and run in a 0.25-cm^3 "artificial straw" (I.M.V. Cassou, France) serving for the deep-freezing of bull semen and filled with the thawed bovine cervical mucus completely,without air bubbles. Ten to 30 min after the start of analysis the "artificial straw" capillaries were frozen, cut into 1-cm pieces, and the activity of capillary pieces was measured in a gamma radiometer with automatic sample shift (NK-350, Gamma Works, Budapest).

In vivo motility studies

Four-month-old virgin rabbits of the New Zealand White breed were subjected to oestrus induction as follows. Two and three weeks before the test the rabbits received 100 IU HCG (Choriogonin inj.) into the ear vein, then another 100 IU dose of HCG was administered 5–10 h before the test (Bockisch, 1993). Only rabbits showing clearly visible oestrous symptoms (swollen, dark red vulva and readiness to mate) were included in the experiment. Before the test, the rabbits were anaesthetized by the intravenous administration of pentobarbital-Na (Nembutal inj. A. U. V., Roche, France) at a dose of 40 mg/kg body mass, then inseminated with 0.5 ml semen containing 2×10^8 radiolabelled spermatozoa of 370 MBq activity. Insemination was carried out with a plastics catheter, in a part of the vagina close to the cervix. The photographs were taken with an MB-9100 type gamma camera (Gamma Works, Budapest) 10, 30, 60, 90 and 120 min after insemination. After taking the photographs, the rabbits were euthanatized and their genitals were exposed. By measuring the activity of the organ parts (using a Capintec CR C-15 R Chamber, Pittsburgh, U.S.A.), the active areas seen on the recordings were identified. The abdominal cavity was rinsed with 500 ml sterile saline, and the activity of the washing fluid was also determined.

A 14-month-old Merino ewe was subjected to oestrus synchronization by the following method. On day 16 before the tests a vaginal sponge (Chronogest, Intervet) was inserted. The sponge was removed on the day before the test. Oestrus was checked using a teaser ram and by vaginoscopy (open cervix). The animal was anaesthetized by intravenous administration of pentobarbital-Na in a dose of 40 mg/kg body mass, then inseminated with 1 ml bull semen containing 3×10^8 spermatozoa of 550 MBq activity, with a plastics catheter inserted 1–2 cm deep into the open cervical orifice. Recordings were made 10, 30 and 120 min after insemination.

Using Tetra S (Bábolna AG, Hungary) laying hybrids in the egg-laying cycle, egg laying was checked by palpation of the abdominal cavity in the afternoon hours, then the layers were anaesthetized by injection of pentobarbital-Na

(30 mg/kg body mass) into the breast muscle. Insemination was performed by inserting a glass catheter into the vagina and introducing 0.5 ml rabbit semen containing 2×10^8 spermatozoa radiolabelled with ^{99m}Tc HM-PAO of 380 MBq activity, or rabbit semen of the same volume and cell count but radiolabelled with ^{111}In-tropolone of 310 MBq activity. Gamma camera recordings were made 10 min, 2 h and 24 h after insemination. Subsequently the birds were euthanatized, the genitals exposed, and the active areas seen on the recordings were identified by measuring the activity of the organ parts. The abdominal cavity was rinsed with 200 ml sterile saline which was also assayed for activity. The viability of spermatozoa recovered in this way was checked by eosin-nigrosin staining.

Results

In vitro motility studies

The aim of the first determinations was to detect differences, if any, between radiolabelled viable spermatozoa, radiolabelled heat-killed spermatozoa and the active radiopharmacon (^{99m}Tc HM-PAO) in the amount and distribution of activity getting into the capillary tube during the run. Fresh rabbit semen and deep-frozen bull semen containing $2.4–3.6\times10^8$ spermatozoa per ml were labelled with one ampoule (0.18 mg HM-PAO) of LEUCO-SCINT kit, with 300 MBq activity. The radiolabelled semen was divided into two parts, and one part was killed in hot water. Another LEUCO-SCINT kit was also labelled with 300 MBq pertechnetate, and the half of it was placed into a plastics container for *in vitro* motility testing. The results show that during a run of viable spermatozoa more than 10 times as much activity gets into the capillary tube as when heat-killed cells are used, and more than twice as much as in the case of ^{99m}Tc HM-PAO. Important differences can be demonstrated also in activity distribution expressed as a function of distance (Figs 1, 2 and 3).

The motility of fresh rabbit and deep-frozen bull spermatozoa was checked 30 min and 3 h after the end of radiolabelling. The spermatozoa were incubated in a water-bath of 37 °C until used. After an incubation of 3 h much lower (about 1/6) total activity was measured in the capillaries and — as regards the activity distribution — appreciable activities were found only in the first cm. No major differences were demonstrable between the different animal species (Figs 4 and 5).

After running fresh rabbit and deep-frozen bull spermatozoa for different times (10, 20 and 30 min), no marked differences were noted in total activity and activity distribution. Nor was there an appreciable difference between the different animal species studied (Fig. 6).

In vivo motility studies

In the rabbit model, the distribution of ^{99m}Tc HM-PAO labelled fresh rabbit and deep-frozen bull spermatozoa was compared at the same points of time. On the polaroid photographs (Figs 7a–e and 8a–e) the migration of homologous spermatozoa seems to be more expressed: from the site of application homologous spermatozoa covered a bigger distance and in larger numbers than heterologous cells. In the case of homologous spermatozoa, retrograde migration is easily distinguishable and after the 30th minute an expressed activity can be detected in the free abdominal cavity. Visualization of the oviducts represents an insolvable technical problem; however, the activity detected in the abdominal cavity provides an indirect proof of their permeability.

In the sheep model the distribution of deep-frozen bull spermatozoa radiolabelled with ^{99m}Tc HM-PAO was studied. Polaroid photographs (Figs 9a–d) were taken 10, 30 and 120 min after insemination. Migration from the site of application was clearly demonstrable already at 30 min. After 120 min, the curved appearance of the left uterine horn was easily distinguishable on the photographs. No visible activity was seen in the abdominal cavity.

The *in vivo* migration of rabbit spermatozoa radiolabelled with ^{99m}Tc HM-PAO and ^{111}In-tropolone was studied in laying hens. No change was found in the activity pattern in the first hours after introduction of spermatozoa into the vagina. However, in the 24th h the entire genital tract could be recognized, and substantial activity was seen in the abdominal cavity as well. During necropsy after euthanasia, the active areas seen in the photographs (Figs 10a–c and 11a–c) were identified by measuring the activity of organ parts. If the animal species was properly chosen, the entire genital tract could be recognized clearly, and substantial activity could be measured around the cyclic ovaries containing large preovulatory follicles.

Discussion

The labelling of ^{99m}Tc HM-PAO spermatozoa radiolabelled with the LEUCO-SCINT kit proved to be stable. The labelling efficiency was high and the viability of cells (ratio of cells not stained by eosin, mass motility as assessed under a stereomicroscope) was acceptable. *In vitro* motility testing was performed as a functional test of labelled spermatozoa.

The active motility of ^{99m}Tc HM-PAO labelled spermatozoa was confirmed by several tests. During the assessment of mass motility under a stereomicroscope, no marked difference was found in comparison to spermatozoa subjected to the labelling procedure but remaining nonlabelled. When examining the capillary tubes under microscope after a 10-min run during the *in vitro* motility test, ^{99m}Tc HM-PAO spermatozoa were found up to a height of 4–5 cm, like in the case of unlabelled spermatozoa.

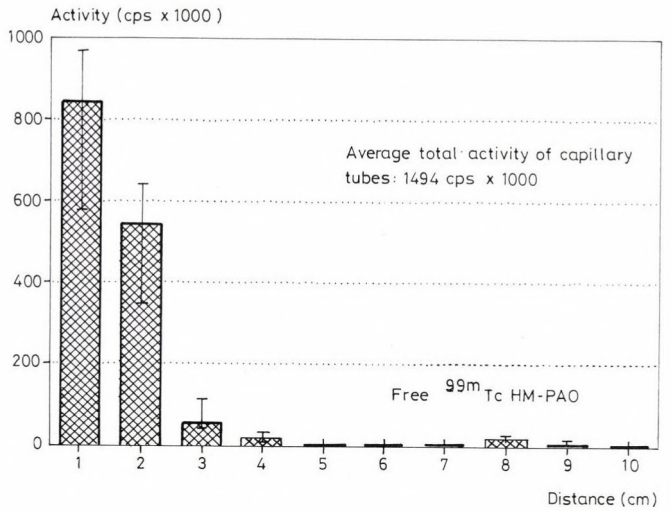


Fig. 1. In vitro motility test of fresh rabbit spermatozoa radiolabelled with ^{99m}Tc HM-PAO. The motility test was started immediately after cell labelling and was performed with a cell count of 1.2–1.8 × 10^8 per ml and an activity of approx. 80 MBq (n=16)

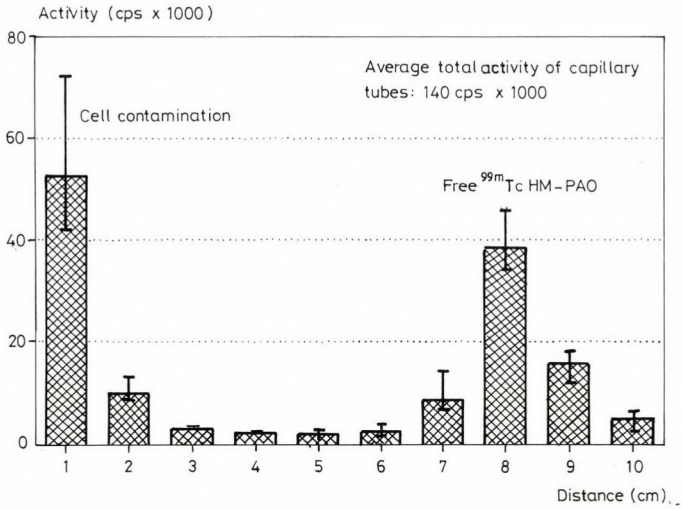


Fig. 2. In vitro motility test of heat-killed fresh rabbit spermatozoa radiolabelled with ^{99m}Tc HM-PAO. After cell labelling, ^{99m}Tc HM-PAO labelled spermatozoa were killed in hot water, then a motility test was performed with a cell count of 1.2–1.8 × 10^8 per ml and an activity of approx. 80 MBq for 10 min (n=16)

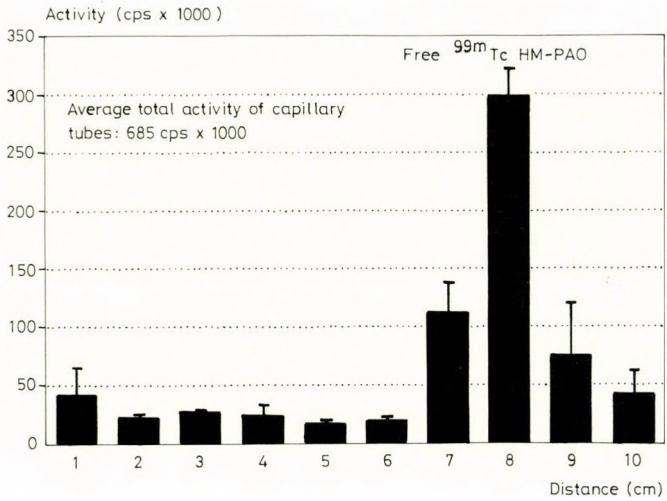


Fig. 3. In vitro motility test of ^{99m}Tc HM-PAO (control test). The labelling substance was run in a volume of 1 ml, with an activity of approx. 80 MBq for 10 min (n=16)

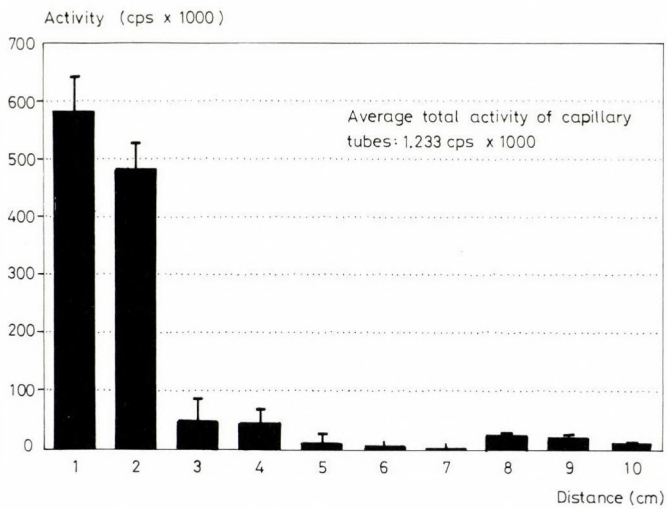


Fig. 4. In vitro motility test of deep-frozen bull spermatozoa radiolabelled with ^{99m}Tc HM-PAO after incubation for 30 min. The radiolabelled cells were incubated in a water-bath of 37 °C for 30 min. The motility test was performed with a cell count of 1.8–2.2 × 10^8 per ml and an activity of approx. 100 MBq for 10 min (n=7)

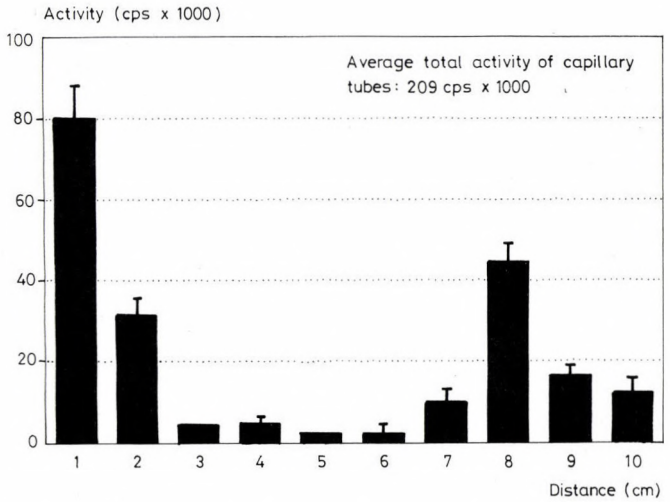


Fig. 5. In vitro motility test of deep-frozen bull spermatozoa radiolabelled with ^{99m}Tc HM-PAO after incubation for 3 h. The radiolabelled cells were incubated in a water-bath of 37 °C for 3 h. The motility test was performed with a cell count of 1.8–2.2 × 10^8 per ml and an activity of approx. 100 MBq for 10 min (n=7)

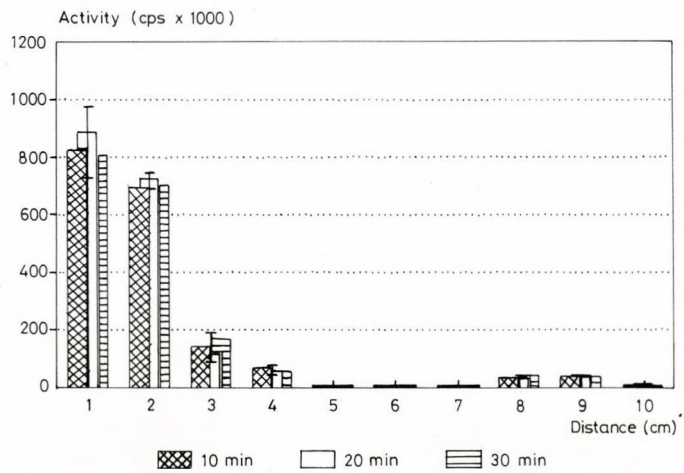
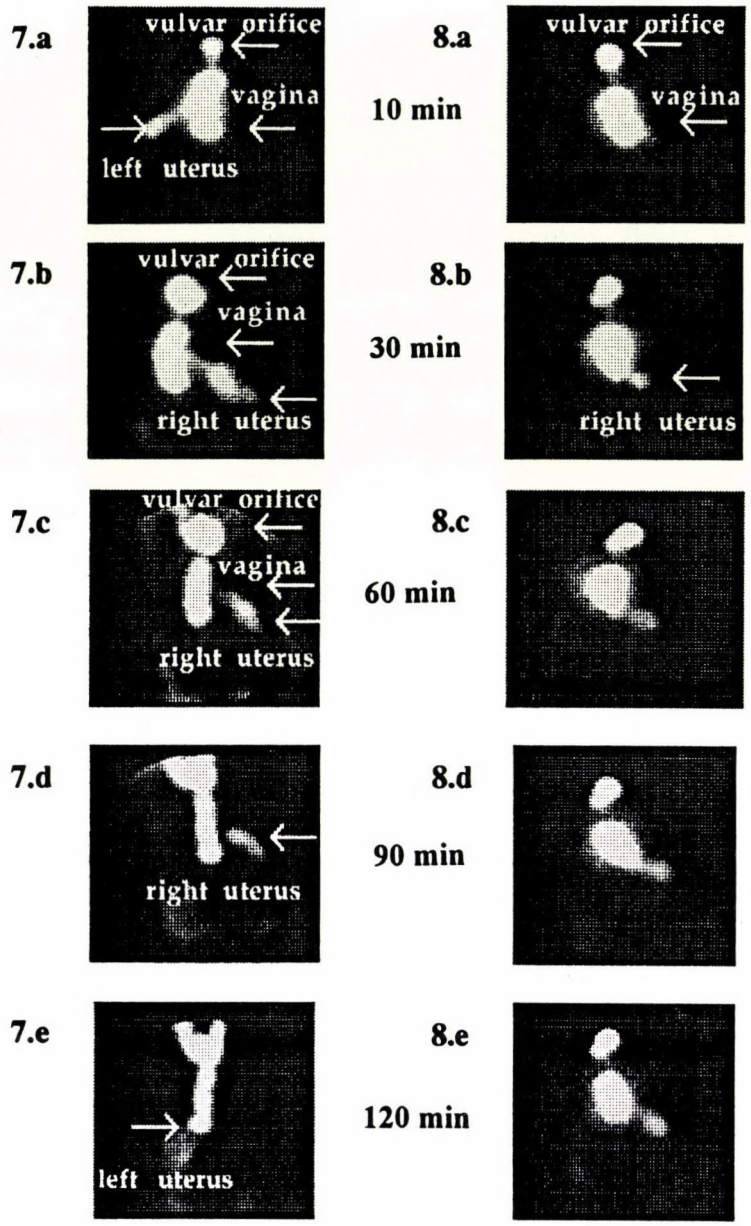


Fig. 6. In vitro motility test of deep-frozen bull spermatozoa radiolabelled with ^{99m}Tc HM-PAO using runs of different duration. The motility test was performed immediately after labelling, with a cell count of 1.4–1.8 × 10^8 per ml and an activity of approx. 80 MBq



Figs 7–8. Migration of 2×10^8 rabbit (1) and bull (2) spermatozoa radiolabelled with ^{99m}Tc HM-PAO in the genital tract of female rabbits. Initial activity: 370 MBq/0.5 ml

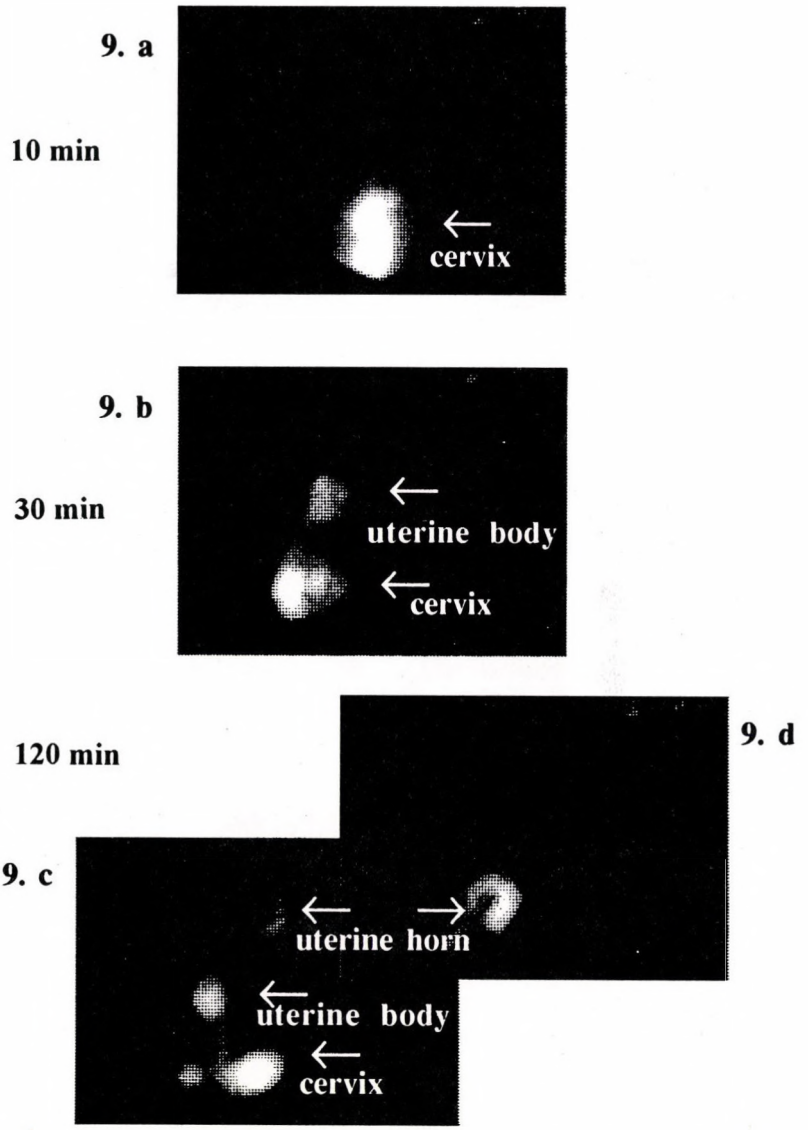
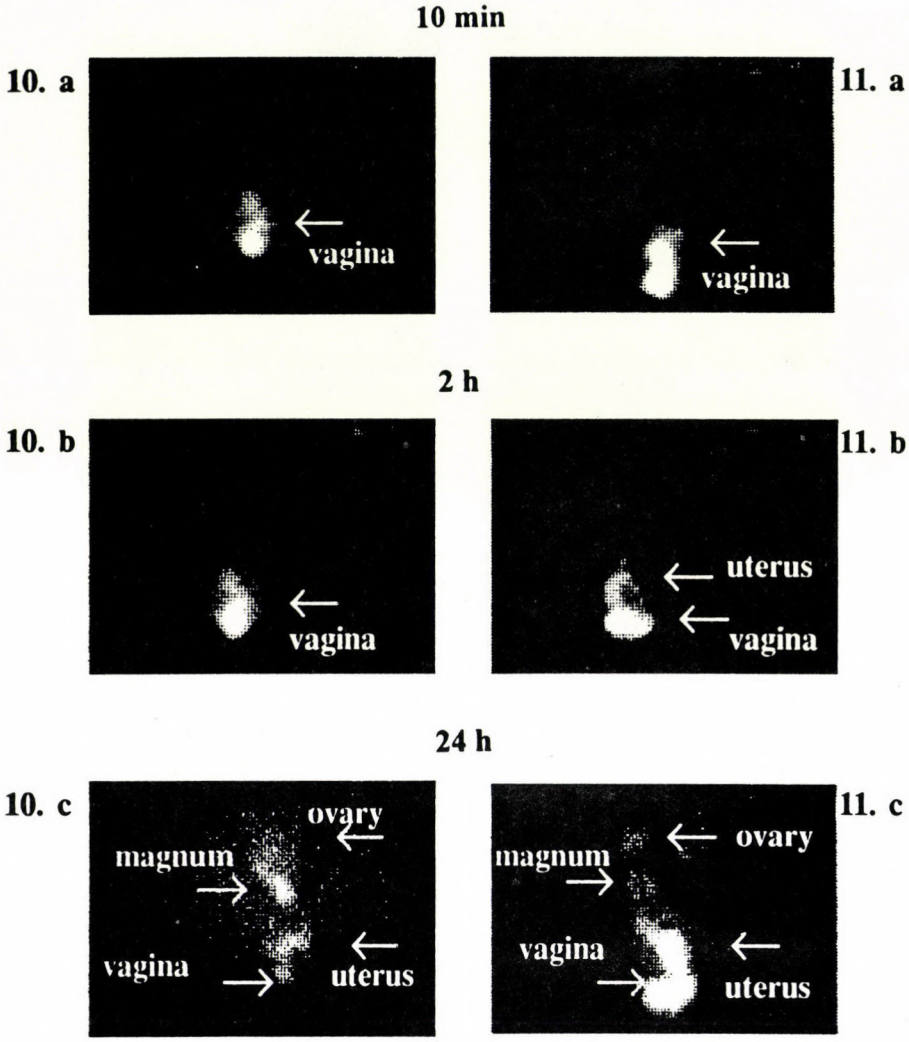


Fig 9. Migration of deep-frozen bull spermatozoa radiolabelled with ^{99m}Tc HM-PAO in the genital tract of female sheep. Cell count: 3×10^8 per ml, activity: 550 MBq



Figs 10–11. Migration of rabbit spermatozoa radiolabelled with ^{99m}Tc HM-PAO and ^{111}In-tropolone in the genital tract of female chicken. Cell count: 2×10^8 per 0.5 ml, activity: 380 MBq ^{99m}Tc and 310 MBq ^{111}In

Activity determination of viable and heat-killed spermatozoa and the radiopharmacon ^{99m}Tc HM-PAO revealed that in the case of viable spermatozoa the activity measured in the capillary tubes was much higher than, and its distribution was markedly different from, the values measured in the other two cases.

No inter-species differences could be demonstrated by running cells from different species for varying times; however, in tests of longer duration the labelled

spermatozoa covered a bigger distance in the capillary tube and in larger numbers. The results of tests conducted after varying incubation times show that ^{99m}Tc HM-PAO spermatozoa must be subjected to motility tests within maximum 2 h (ideally within 0.5 h) after the labelling procedure.

In vivo tests using the rabbit model proved that homologous spermatozoa migrated to a bigger distance from the site of application and in larger numbers as compared to heterologous spermatozoa (deep-frozen bull sperm) during the same period of time. The scintigrams made by us show good agreement with the scintiscans published by Bockisch et al. (1989) on the distribution of radioactivity. However, based upon the activity measurements in organ parts of animals necropsied after euthanasia, we — in contrast with the above-mentioned authors — claim that this method is not suitable for the visualization of spermatozoa staying in the oviduct. However, the large number of spermatozoa getting into the abdominal cavity provides indirect evidence that the oviduct is permeable to male germ cells.

Using heterologous spermatozoa (deep-frozen bull semen) for insemination in the sheep model, we could not find substantial activity in the oviduct. This is in contrast with the observations reported by Lorton et al. (1980). There was no visible activity in the abdominal cavity either. The curved appearance of the left uterine horn on the photographs indicates the presence of a large mass of spermatozoa.

The investigations with domestic hen provided useful information for almost all details of the genital tract. Twenty-four h after insemination, large numbers of heterologous (fresh rabbit) spermatozoa labelled in two different ways were present in the vagina, uterus, magnum and in the periovarian part of the abdominal cavity.

Summing up the results of experiments conducted on three animal species, it can be stated that spermatozoa labelled with ^{99m}Tc HM-PAO are suitable for the determination and visualization of sperm cell distribution in the genital organs of live female animals by a noninvasive, bloodless procedure. In certain cases of infertility of female animals, the method seems to be suitable for studying the permeability of the oviduct as well. The latter examination would be rendered especially useful by the fact that the information provided by it would characterize the permeability of the oviduct to spermatozoa, in contrast with the hitherto applied X-ray contrast examinations and other scintigraphic methods using exclusively radioactive solutions (Iturralde and Venter, 1981; Stone et al., 1985). The high *in vivo* stability of ^{99m}Tc HM-PAO labelled spermatozoa is demonstrated by the fact that the image of the thyroid, the gastric mucosa or other organs — as a sign of free radiopharmacon or ^{99m}Tc-pertechnetate — appeared on none of the photographs taken by us at the test intervals used and in none of the animal species included in the study.

Acknowledgements

This work was supported by grant ETT 381/1990 and grant no. 2028 of the National Research Fund (OTKA). We are indebted to Dr. István Perjés (Pannon University of Agricultural Sciences, Kaposvár) for making available to us the *in vitro* motility analyzer. We thank Mrs L. Dirner and Ms Zsuzsanna Suhajda for their valuable help in the practical works and for careful preparation of the manuscript.

References

Ballinger, J. R., Reid, R. M. and Gullenchyn, K. Y. (1988): Radiochemical purity of (99m Tc) HMPAO. J. Nucl. Med. **29,** 572–576.

Balogh, L., Szász, F., Jánoki, Gy. A., Zöldág, L. and Huszenicza, Gy. (1992): Labelling of spermatozoa with ^{99m}Tc HM-PAO: Optimization of the labelling conditions for a LEUCO-SCINT kit (in Hungarian). Izotóptechnika, Diagnosztika **35,** 91–96.

Becker, W., Borst, U., Krahe, T. and Börner, W. (1989): Tc-99m-HMPAO labelled human platelets: *in vitro* and *in vivo* results. Eur. J. Nucl. Med. **15,** 296–301.

Becker, W. and Börner, W. (1988): 99mTc-HMPAO zur Markierung von Leucozyten und Thrombozyten. Besonderheit und Diagnostische Konsequenzen. Fortsch. Röntgenstr. **148,** 69–74.

Becker, W., Börner, W., Borst, U., Kromer, E. P. and Gruner, K. R. (1987): Tc-99m-HMPAO: A new platelet labeling compound? Eur. J. Nucl. Med. **13,** 267–268.

Biersack, H. J., Linke, D., Brassel, F., Reichman, K., Kurthen, M., Durwen, H. F., Reuter, B. M., Wappenschmidt, J. and Stefan, H. (1987): Technetium-99m HM-PAO brain spect in epileptia patients before and during unilateral hemispheric anaesthesia (Wada test): Report of three cases. J. Nucl. Med. **28,** 1763–1767.

Bockisch, A. (1990): Radionuklid-Markierung Menschlicher und Tierischer Spermatozoan und Quantitative Szintigraphie der Spermatozoenkinetik im Genitaltract des Weiblichen Kaninchens nach Insemination. Habilitationsschrift, Klinik für Nuklearmedizin der Universität Bonn, 1990.

Bockisch, A. (1993): Sperm cell dynamics in the female rabbit genital tract after insemination monitored by radiolabeled spermatozoa. J. Nucl. Med. **34,** 1134–1139.

Bockisch, A., Al-Hasani, S., Ven, H. V. D., Diedrich, K., Posch, Ch., Hotze, A., Krebs, D. and Biersack, H.-J. (1989): Markierung von Spermatozoen mit 99mTc-HMPAO und *in vivo* Darstellung der Spermatozoenmigration beim Kaninchen. Nuclear Compact **20,** 65–70.

Danpure, H. J. and Osman, S. (1988): Investigations to determine the optimum conditions for radiolabeling human platelets with 99Tcm-hexamethylpropylene amine oxime (99Tcm-HMPAO). Nucl. Med. Commun. **9,** 267–272.

Danpure, H. J., Osman, S. and Carroll, M. K. (1986): *In vitro* studies of a clinical protocol for radiolabeling mixed leucocytes with 99mTc-HMPAO. Nucl. Med. Commun. **8,** 280–286.

Dewanje, M. K., Shyam, A. R. and Didisheim, P. (1984): Indium-111 tropolone, a new high-affinity platelet label: Preparation and evaluation of labelling parameters. J. Nucl. Med. **22,** 981–987.

Iturralde, M. and Venter, P. F. (1981): Hysterosalpingo-radionuclide scintigraphy (HERS). Seminars in Nuclear Medicine **11,** 301–314.

Kummerfeld, H. L., Lorton, S. P. and Foote, R. H. (1981): Relationship of spermatozoal migration in cervical mucus to bovine fertility. J. Androl. **2,** 103–107.

Kung, H. F. and Blau, M. (1980): Regional intracellular pH shift: a proposed new mechanism for radiopharmaceutical uptake in brain and other tissues. J. Nucl. Med. **21,** 147–152.

Langen, K. J., Roosen, N., Herzog, H., Kuwert, T., Kiwit, I. C. W., Bock, W. J. and Feinendegen, L. E. (1989): Investigations of brain tumours with 99Tcm-HM PAO SPECT. Nucl. Med. Commun. **10,** 324–329.

Lorton, S. P., Gatley, S. J., Liebermann, L. M. and First, N. L. (1980): Radiolabelling of mammalian spermatozoa and their use to monitor sperm transport in females. Am. J. Obstetr. Gynaecol. **136,** 732–736.

Oliphant, G., Singhas, C. A., Thomas, T. S. and Suarez, S. S. (1977): Iodination of the plasma membrane of rabbit sperm. Society for the Study of Reproduction, Austin, Texas, 1977. Abstr. 89.

Olive, D. L., Weinberg, J. B. and Haney, A. F. (1987): Sperm-macrophage interaction in the mouse: A quantitative assay *in vitro* using ^{111}In oxin-labelled sperm. Biol. Reprod. **37,** 1170–1178.

Overstreet, J. W. and Tom, R. A. (1982): Experimental studies of rapid sperm transport in rabbits. J. Reprod. Fert. **66,** 601–606.

Papós, M., Láng, J., Csernay, L., Lázár, J. and Jánoki, Gy. A. (1989): Leucocyte labelling with an own-produced d,l HMPAO preparation that can be labelled with 99Tcm: Factors affecting labelling efficiency (in Hungarian). Izotóptechnika, Diagnosztika **32,** 9–16.

Peters, A. M., Danpure, H. J., Osman, S., Hawker, R. J., Henderson, B. L., Hodgson, H. J., Kelly, J. D., Neinrinckx, R. D. and Lavender, J. P. (1986): Clinical experience with 99mTc-hexamethylpropylene-amine-oxime for labelling leucocytes and imaging inflammation. Lancet **ii,** 946–951.

Podreka, I., Suess, E., Goldenberg, G., Steiner, M., Brücke, T., Müller, Ch., Lang, W., Neinrickx, R. D. and Deecke, L. (1987): Initial with Technetium-99m HM-PAO brain SPECT. J. Nucl. Med. **28,** 1657–1666.

Smith, T. T., Koyanagi, F. and Yanagimachi, R. (1987): Distribution and number of spermatozoa in the oviduct of the golden hamster after natural mating and artificial insemination. Biol. Reprod. **37,** 225–234.

Stone, S. C., McCalley, M., Braunstein, P. H. and Egbert, R. (1985): Radionuclide evaluation of the tubal function. Fert. Steril. **43,** 757–760.

Suttiyotin, P. and Thwaites, C. J. (1992): Comparison of a swim-up technique with the Hamilton Thorn motility analyser for measurement of sperm velocity and motility. Reprod. Fert. Developm. **4,** 153–160.

Templeton, A. A. and Mortimer, D. (1980): Laparascopic sperm recovery in infertile women. Br. J. Obstetr. Gynaecol. **87,** 1128–1134.

Young, R. J., Bodt, B. A., Iturralde, T. G. and Starke, W. C. (1992): Automated analysis of rabbit sperm motility and the effect of chemicals on sperm motion parameters. Mol. Reprod. Developm. **33,** 347–356.

Acta Veterinaria Hungarica 43 (1), pp. 79–87 (1995)

EFFECTS OF SAMPLE HANDLING TEMPERATURES ON BOVINE SKIM MILK PROGESTERONE CONCENTRATIONS

H. M. EISSA*, R. F. NACHREINER and K. R. REFSAL

Endocrine Diagnostic Laboratory Section, Animal Health Diagnostic Laboratory, Michigan State University, East Lansing, MI 48823, U. S. A.

(Received January 5, 1994)

The effect of incubation of whole milk at various temperatures and times on the amount of progesterone (nmol/l) in the skim milk fraction was determined. For this study, milk samples were obtained from 10 pregnant Holstein cows. The whole milk samples were incubated at 37 °C (near normal body temperature of the cow) for 4 h and the initial skim milk progesterone concentration was determined. After that, the experiment was carried out in two main steps: (I) The test tubes containing the whole milk were divided into 4 groups and incubated at different temperatures (0, 4, 20 and 37 °C). Samples were removed at 30, 60, 90 and 120 min. (II) After 120 min of incubation at different temperatures, the remaining test tubes were returned to the water bath at 37 °C for another 30, 60 and 90 min. The initial average of skim milk progesterone concentrations after incubating the whole milk at 37 °C for 4 h was 11.0 ± 4.4 nmol/l. When the whole milk was incubated at 0 °C , the skim milk progesterone concentration increased ($P < 0.05$) to 14.6 nmol/l at 30 min and reached 16.2 nmol/l at 60 min of incubation. At 4 °C incubation temperature, skim milk progesterone increased significantly ($P < 0.05$) to 15.3 nmol/l and reached 16.9 nmol/l after 90 min. When the whole milk was left at 20 °C, the initial skim milk progesterone values decreased to 9.5 nmol/l after 30 min incubation and no further decreases were found even if the whole milk was returned to 37 °C for 90 min. After incubating the whole milk in a water bath at 37 °C, the skim milk progesterone dropped significantly ($P < 0.05$) at 120 min to 8.6 nmol/ml and no further changes were found. Whole milk incubated at 0 °C and 4 °C had the greatest changes in the concentrations of progesterone in the skim milk. When these samples were returned to 37 °C for 90 min, progesterone dropped to concentrations not significantly different from the precooling concentrations within 30 min. From this study, it can be concluded that skim milk progesterone concentration is temperature dependent and sample handling can result in profound changes in the amount of progesterone in various fractions. The solubility of progesterone in milk fat may cause this temperature-dependent phenomenon.

* Dr. Eissa's present address is: Theriogenology Department, Faculty of Veterinary Medicine, Cairo University, Giza, 12211 Egypt

0236–6290/95/$ 4.00 ©1995 *Akadémiai Kiadó, Budapest*

Key words: Bovine, milk, skim milk, progesterone, sample handling, temperature

Measurement of circulating reproductive hormone levels provides a useful tool for studying ovarian status and diagnosing fertility problems in domestic animals. The decline or rise may reflect functional changes in the reproductive system. Measurement of progesterone can be used to monitor the functional capacity of the corpus luteum. Clinically, progesterone levels may also be used for pregnancy determination and in the diagnosis of some reproductive endocrine disorders. The assayed level of progesterone is not only affected by the physiological condition of the animals but also by sample handling. This may influence results more than the accuracy of the radioimmunoassay which is used (Fahmi et al., 1985).

However, in blood samples, progesterone fell to non-detectable levels after two days at room temperature and significant declines occurred even if the blood was refrigerated, or centrifuged but left with the red blood cells (Oltner and Edqvist, 1982). The rate of progesterone degeneration was temperature dependent. Even when the blood samples were kept at refrigeration temperatures prior to their centrifugation some progesterone were lost. This was due to *in vitro* degeneration of progesterone by the blood cells (Fahmi et al., 1985). Vahdat et al. (1979) examined the effect of incubating whole blood of pregnant cows with or without EDTA at 4 °C for 6 or 24 h. They concluded that time and temperature of handling whole blood before separating plasma or serum significantly affected concentrations of progesterone. A similar study was conducted by Owens et al. (1980) using blood samples taken from cows during the luteal phase of the oestrous cycle. In their study, progesterone measured in plasma decreased significantly after 3 h, and after 24 h it decreased by 75%. Serum levels of progesterone did not decrease until 12 h after incubation and after 24 h they decreased to 3% of the original values.

Milk progesterone radioimmunoassay offers considerable advantages over plasma because milk is easier to obtain from milking cows than blood. Progesterone may be measured in whole milk (Lamming and Bulman, 1976), fat-free milk (Pope et al., 1976) or in milk fat (Claus and Rattenberger, 1979) and although each technique gives different concentrations (Bulman, 1979), the overall interpretation was similar. Nachreiner et al. (1986) reported that milk progesterone concentrations vary widely due to sample handling, species, environmental conditions and assay differences. Assaying the skim milk fraction or fat fraction can elevate the error which is introduced by various fat percentages and the concern about whether the samples are from fore milk, a composite sample, or strippings is negated (Oltner and Edqvist, 1981). However, Abdel Rahim et al. (1982) found that the progesterone concentrations in cow's whole milk were 6.26 ng/ml before suckling and 27.7 ng/ml after suckling. These differences are not negligible. Heap et al. (1975) reported that milk fat contained 80% of 3H-progesterone, ca-

sein had 19% and the whey had 1%, indicating some protein binding of the progesterone.

Although several reports have suggested that time and temperature of handling blood between collection and centrifugation affect the levels of progesterone measured in bovine blood samples, there was only one report discussing the effects of time and temperature of incubation on concentrations of milk progesterone (Nachreiner et al., 1992). The focus of this study was to quantify the effects of incubation times and temperatures on milk progesterone concentrations and to determine whether these changes are reversible.

Materials and methods

Experimental animals. For this study, 10 pregnant Holstein cows aged 4–6 years were used. The animals were kept at a dairy barn of Michigan State University. Pregnancy was determined according to the insemination dates and confirmed by rectal examination.

Sampling procedures. Milk samples (100 ml) were collected from each cow into sterile plastic bags (Whirl-Pak, Nasco) and transported directly to the laboratory. Each sample was divided into several portions in polypropylene test tubes (12 × 75 mm). The tubes were incubated in a water bath at 37 °C for 4 hours. After that, the experiment was carried out in two major steps: (1) The test tubes containing the whole milk were divided into 4 groups and incubated at different temperatures [0 °C (in crushed ice container), 4 °C (in water bath at 4 °C), 20 °C (at room temperature) and 37 °C (in water bath at 37 °C)]. Samples were removed at 30, 60, 90 and 120 min. The whole milk was centrifuged for 10 min at 4,229 × g, the skim milk was pipetted into a second 12 × 75 mm test tube and stored at –20 °C until analysis on the next day. (2) After 120 min of incubation at the different temperatures, the remaining test tubes were transferred back to the water bath at 37 °C for another incubation of 30, 60 and 90 min. The whole milk was also centrifuged and processed as above.

Progesterone assay. Progesterone (P4) was assayed without extraction from skim milk by using the FAO-IAEA solid phase kit (Coat-a-Count, Diagnostic Products Corporation). Standards (0 to 64 nmol/l) were made in skim milk in order to avoid a difference in assay protein matrix. The sensitivity of the assay based upon 10% suppression of binding of the assay curve was 0.3 nmol/l. The specificity of the progesterone assay as reported by the commercial company is shown in Table 1. No closely related progestins had significant cross-reactivity. The interassay coefficients of variation averaged 9.6% at 3.8 nmol/l and 5.7% at 16.9 nmol/l (n = 9). The intraassay coefficients of variation averaged 3.8% at

3.8 nmol/l and 3.4% at 16.9 nmol/l (n = 9). The accuracy based on recovery of added progesterone was 98.7% at 15.9 nmol/l and 100.5% at 31.8 nmol/l.

Table 1

Cross-reactivity of steroids in the progesterone assay

Compound	Approximate per cent of cross-reactivity
Progesterone	100%
Corticosterone	0.4%
11-Deoxycorticosterone	1.7%
11-Deoxycortisol	2.4%
20α-Dihydroprogesterone	2.0%
17α-Hydroxyprogesterone	0.3%
B-Pregnan-3α, -ol-20-one	0.2%
5α-Pregnan-3, 20-dione	0.8%
5B-Pregnan-3, 20-dione	1.3%

Analysis of the data. Analysis of variance (SAS User's Guide) was used to assess the effects of post-insemination interval on milk progesterone. The first analysis tested differences among days from 0 to 45. The second analysis tested whether luteal concentrations of milk progesterone changed throughout gestation until lactation ceased. For this analysis, the post-insemination days were divided into 12 periods that covered 2-week intervals. Contrasts among periods were made with Scheffe's interval (Gill, 1978).

Results

The effect of incubation of whole milk at various temperatures and times on the amount of progesterone (nmol/l) in the skim milk fraction is shown in Table 2 and Fig. 1. The initial average of progesterone concentrations in skim milk (11.0 ± 4.4 nmol/l) was determined after keeping the whole milk at 37 ° C (near normal body temperature of the cow) for 4 h.

When the whole milk was incubated at 0 °C the concentration of progesterone in the skim milk fraction increased ($P < 0.05$) to 14.6 nmol/l at 30 min and reached 16.2 nmol/l at 60 min of incubation. No further significant increase occurred during the next hour. At 4 °C incubation temperature, progesterone in

Table 2

Effect of incubation of whole milk at various temperatures and times on the amount of progesterone (nmol/l) in the skim milk fraction

Temperature of first incubation	Minutes of incubation					Minutes after return to 37 °C		
	0*	30	60	90	120	30	60	90
0 °C	$11.1^{a(I)}$	$14.6^{b(II)}$	$16.2^{c(II)}$	$16.5^{c(II)}$	$16.5^{c(II)}$	$10.5^{a(I)}$	$11.1^{a(II)}$	$10.5^{a(II)}$
4 °C	$11.1^{b(I)}$	$15.3^{cd(II)}$	$14.6^{c(II)}$	$16.9^{d(III)}$	$16.2^{cd(II)}$	$9.2^{a(I)}$	$11.4^{b(II)}$	$10.2^{ab(II)}$
20 °C	$11.1^{b(I)}$	$9.5^{a(I)}$	$9.2^{a(I)}$	$11.1^{b(II)}$	$9.5^{a(I)}$	$10.5^{ab(I)}$	$9.2^{a(I)}$	$9.2^{a(I)}$
37 °C	$11.1^{b(I)}$	$9.5^{ab(I)}$	$9.2^{ab(I)}$	$9.2^{ab(I)}$	$8.6^{a(I)}$	$9.9^{ab(I)}$	$8.9^{a(I)}$	$8.3^{a(I)}$

a, b, c, d = means within a row (temperature) with different alphabetical superscripts are significantly different ($P < 0.05$)
I, II, III = means within a column (time) with different Roman numerical superscripts are significantly different ($P < 0.05$)
* = initial concentrations of progesterone in skim milk after incubation of the whole milk at 37 °C for 4 h

skim milk fraction also significantly increased ($P < 0.05$) to 15.3 nmol/l at 30 min and reached 16.9 nmol/l after 90 min of incubation. No further significant increase occurred. When the whole milk was left at room temperature (20 °C), the initial skim milk progesterone values decreased to 9.5 nmol/l after 30 min of incubation. No further decreases were found even though the whole milk was returned to 37 °C for 90 min. After incubating the whole milk in a water bath at 37 °C, the progesterone level of the skim milk dropped ($P < 0.05$) at 120 min to 8.6 nmol/l and no further significant changes were found.

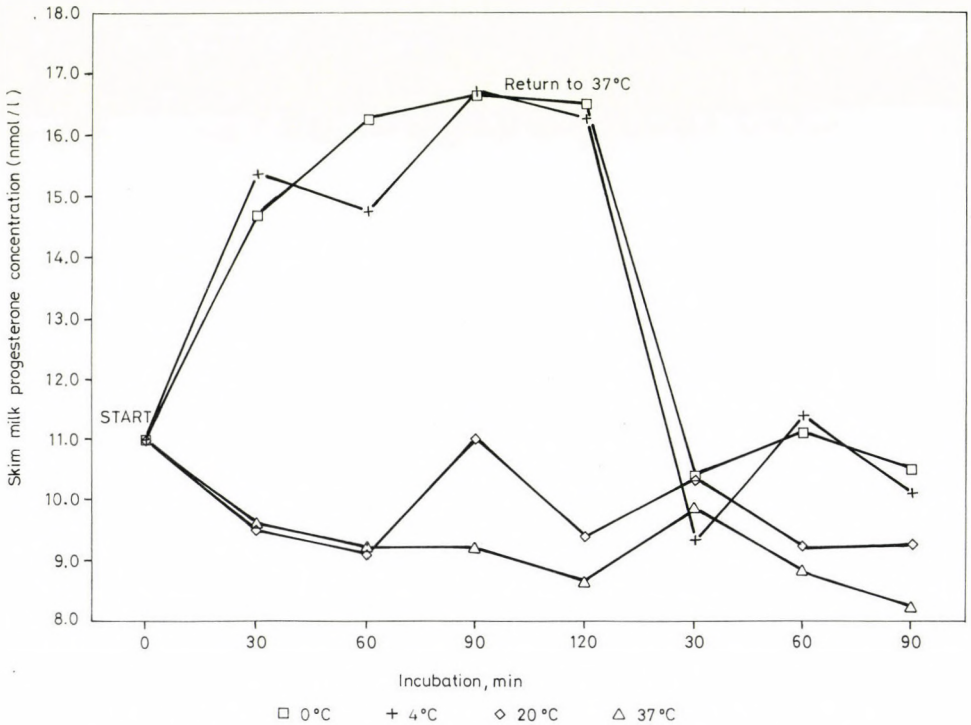


Fig. 1. Effects of time and temperature on progesterone in skim milk

Whole milk incubated at 0 °C and 4 °C had the greatest changes in the concentrations of progesterone in the skim milk fraction. These samples were returned to incubation at 37 °C for 90 min and progesterone dropped to concentrations not significantly different from the precooling levels within 30 min.

Discussion

The data of Nachreiner et al. (1992) indicated that the skim milk fractions of progesterone are dependent upon 2 major factors, the butterfat per cent and the temperature of the whole milk at the time of centrifugation. Whether this is purely a physical principle or dependent upon enzymatic interconversion was not determined.

In studies of whole blood, enzymes play a major role in the loss of progesterone. Van der Molen and Groen (1968) reported glucose-dependent interconversion of progesterone to 20α-hydroxyprogesterone when incubated with human, dog, rabbit or sheep whole blood. In the absence of glucose, this interconversion favoured progesterone. In the absence of cells, progesterone was quite stable (Vahdat et al., 1981; Reimers et al., 1983), indicating that the interconversion is dependent upon cellular enzymes. The progesterone concentration in whole blood could even be increased from the lowest level after storage for 24 h (glucose presumably depleted) (Vahdat et al., 1981). The interconversion was delayed when the blood was cooled to 4 °C. A cellular component is also present in whole milk. The leukocyte count of the present samples was about 25,000 per ml. There is the potential for some enzymatic interconversion of the progesterone in milk, but with so few cells compared to whole blood, that seems remote.

Progesterone is reported to be practically insoluble in water (42.6 μmol/l) but more soluble in serum (1,250 μmol/l). More progesterone is soluble in saline at 37.5 °C than at 25 °C (Bischoff and Pilhorn, 1948). Progesterone is known to be more fat soluble, being soluble in alcohol, acetone, petroleum ether and diethyl ether (Windholz et al. 1976). Whether the solubility is also temperature dependent was not reported, but in most instances it is also a significant factor.

Progesterone rapidly increased in the aqueous fraction of milk upon cooling (Nachreiner et al., 1992), a physical principle which is the opposite of that reported for increased solubility of progesterone in aqueous solutions as the temperature was increased (Bischoff and Pilhorn, 1948). Whether this interconversion is enzyme dependent has not been fully elucidated; however, the increase in the aqueous fraction upon cooling would also be the opposite of what one would expect of an enzyme. For instance, enzymatic interconversion was slowed upon cooling of blood. If enzymatic interconversion was involved in the present study one would expect the progesterone to be higher at the first times of incubation and show the greatest changes (decreases) at the higher temperatures as was found with whole blood. This did not happen. The lack of change at 37 and 20 °C and the increases in skim milk progesterone at 4 and 0 °C are not in agreement with the enzymatic changes which occur in whole blood.

Milk fat was found to be a highly significant component of the prediction equation reported by Nachreiner et al. (1992). A possible explanation of the dif-

ferences in skim milk progesterone concentrations is a purely physical principle based on the temperature-dependent solubility of progesterone in milk fat. In the present study, as milk fat was cooled, the progesterone became less soluble and was forced out of the fat fraction into the aqueous fraction where it was still quite soluble. Then, as the whole milk was heated again, the progesterone was solubilized into the fat fraction from the aqueous fraction.

These data are important to consider when obtaining milk samples from dairy cows. Sample handling can result in profound changes in the amount of prgesterone in various fractions. If the milk is cooled more will be found in the skim milk and as the milk is warmed more will be found in the fat fraction. It is very important to be consistent in sample processing and always centrifuge the samples at the same temperatures during a study. In addition, if levels are published for clinical decision making, the sample handling must be included to be certain that misdiagnoses are avoided. Cows that are pregnant may be misdiagnosed as open if the skim milk normal concentrations were determined with cold samples but a clinical sample was warm at the time of centrifugation. Fore milk, mid milking and skim milk are also significant components which can affect the concentration of progesterone in whole and skim milk samples as reported previously (Nachreiner et al., 1986). These factors should also be kept constant in sample handling for increased accuracy.

Acknowledgements

The authors wish to acknowledge the help of Dr. T. Herdt and Mr. Bob Kreft. We also acknowledge the support from the Peace Fellowship Program, USAID and the Egyptian Cultural and Educational Bureau, Washington, DC and the Animal Health Diagnostic Laboratory, Michigan State University, East Lansing, MI.

References

Abdel Rahim, S. E. A., Lowman, B. G., Deas, D. W., Jeffery, F. H. and Hunter, E. A. (1982): The effect of time of sampling in relation to time of suckling on milk progesterone levels in beef cows. Br. Vet. J. **138**, 409–416.

Bischoff, F. and Pilhorn, H. R. (1948): The state and distribution of steroid hormones in biologic systems. III. Solubilities of testosterone, progesterone and α-estradiol in aqueous salt and protein solution and in serum. J. Biol. Chem. **174,** 663–682.

Bulman, D. C. (1979): The measurement of progesterone in milk. Br. Vet. J. **135,** 460–469.

Claus, R. and Rattenberger, E. (1979): Improved method for progesterone determination in milk-fat. Br. Vet. J. **135,** 464–469.

Fahmi, H. A., Williamson, N. B., Tibary, A. and Hegstad, R. L. (1985): The influence of some sample handling factors on progesterone and testosterone analysis in goat. Theriogenology **24,** 227–233.

Gill, J. L. (1978): Design and Analysis of Experiments in the Animal and Medical Sciences, First Edition. The Iowa State University Press, Ames, Iowa 50010. Vol. 1, pp. 177–178.

Heap, R. B., Henville, A. and Linzell, J. L. (1975): Metabolic clearance rate, production rate, and mammary uptake and metabolism of progesterone in cows. J. Endocr., 66: 239–247.

Lamming, G. E. and Bulman, D. C. (1976): The use of milk progesterone radioimmunoassay in the diagnosis and treatment of subfertility in dairy cows. Br. Vet. J. **132,** 507–517.

Nachreiner, R. F., Oschmann, S. J., Edqvist, L. -E. and Richards, J. I. (1986): Activities of the FAO/IAEA animal production unit of the IAEA Seibersdorf Laboratory. Part I: Solid-phase radioimmunoassay (RIA) approciate for use in developing countries. In: Nuclear and Related Techniques in Animal Production and Health (Proceedings of a Symposium, Vienna, 17–21 March). pp. 653–659.

Nachreiner, R. F., Oschmann, S. J., Edqvist, L. -E. and Richards, J. I. (1992): Factors affecting skim milk progesterone assay results. Am. J. Vet. Res. **53,** 1085–1089.

Oltner, R. and Edqvist, L. -E. (1981): Progesterone in defatted milk: its relation to insemination and pregnancy in normal cows as compared with cows on problem farms and individual problem animals. Br. Vet. J. **137,** 78–87.

Oltner, R. and Edqvist, L. -E. (1982): Changes in plasma progesterone levels during storage of heparinized whole blood from cow, horse, dog and pig. Acta Vet. Scand. **23,** 1–8.

Owens, R. E., Atkins, D. T. and Rahe, C. H. (1980): Time-dependent loss on radioimmunoassayable level of progesterone following ambient temperature incubation of heparinized bovine blood. Theriogenology **4,** 305–309.

Pope, G. S., Majzlik, I., Ball, P. J. H. and Leaver, J. D. (1976): Use of progesterone concentration in plasma and milk in the diagnosis of pregnancy in domestic cattle. Br. Vet. J. **132,** 497–506.

Reimers, T. J., McCann, J. P. and Cowan R. G. (1983): Effects of storage times and temperatures on T3, T4, LH, prolactin, insulin, cortisol and progesterone concentrations in blood samples from cows. J. Anim. Sci. **57,** 683–691.

Vahdat, F., Hurtgen, J. P., Whitmore, H. L., Johnston, S. D. and Ketelsen, C. L. (1979): Effect of time and temperature on bovine serum and plasma progesterone concentration. Theriogenology **12,** 371–374.

Vahdat, F., Hurtgen, J. P., Whitmore, H. L. Seguin, B. E. and Johnston, S. D. (1981): Decline in assayable progesterone in bovine plasma: effect of time, temperature, anticoagulant and presence of blood cells. Am. J. Vet. Res. **42,** 521–522.

Van der Molen, H. J. and Groen, D. (1968): Interconversion of progesterone and 20a-dihydroprogesterone and of androstenedione and testosterone *in vitro* by blood and erythrocytes. Acta Endocrinol. **58,** 419–444.

Windholz, M., Budavari, S., Stroumtsos, L. Y. and Fertig, M. N. (1976): The Merck Index. Merck and Co., Inc., Rahway, N. J., U. S. A., p 1007.

Acta Veterinaria Hungarica 43 (1), pp. 89–93 (1995)

EFFECT OF LUTEINIZING HORMONE AND ESTRADIOL ON *IN VITRO* MATURATION OF FOLLICULAR OOCYTES IN GOAT (*Capra hircus*)

K. S. SIDHU and S. CHEEMA

Andrology Laboratory, Department of Zoology, Punjab Agricultural University, Ludhiana 141 004, India

(Received January 5, 1994)

In vitro maturation of goat follicular oocytes in TALP medium supplemented with variable concentrations of luteinizing hormone (LH) [5–20 μg/ml] and estradiol [1 μg/ml] was studied. Incubation of viable oocytes was carried out at 38.5 °C under liquid paraffin in 5% carbon dioxide/air mixture for 24 h. At 0 h all the oocytes were in germinal vesicle (GV) stage, each with a good cumulus. The cumulus expansion increased linearly with increasing concentrations of LH and a fixed concentration of estradiol [1 μg/ml] at 24 h of incubation. Germinal vesicle breakdown (GVBD) was observed in 28% of the oocytes at 5 μg/ml concentration of LH and the same increased to 87% at 10 μg/ml of LH with 13% oocytes at metaphase II. At 20 μg/ml of LH, 45% of the oocytes were at metaphase II and the rest were at metaphase I. LH and estradiol seem to be important for acquisition of maturation competence in goat follicular oocytes.

Key words: Goat, follicular oocyte, germinal vesicle, cumulus, metaphase II, polar body

Spontaneous meiotic maturation occurs if the oocytes are removed from the follicles. However, the subsequent development seldom progresses beyond the first few cleavage divisions in most mammalian species (Moor, 1978; Eppig and Downs, 1988; Cross and Brinster, 1990). Moor and Trounson (1977) described that oocytes cultured within the follicle in a hormone-free medium remained at the germinal vesicle stage unless supplemented with follicular stimulating hormone (FSH) and LH, whereupon these oocytes reached the second meiotic division. Subsequently several studies demonstrated the beneficial effects of various hormones such as progesterone, estradiol, FSH, LH on *in vitro* maturation of follicular oocytes in many mammalian species (Bar Ami and Tsafriri, 1981; Karlach, 1986; Eppig and Downs, 1988; Zhang and Armstrong, 1989; Zuelka and Brackett, 1990; Frylinck and Kay, 1992). Although some studies have been conducted on the *in vitro* maturation and fertilization of oocytes in sheep and goat (Moor and Trounson, 1977; Pugh et al., 1991; Chauhan and Anand, 1991; Frylinck and Kay, 1992; Smedt et al., 1992), little attention has been paid to the

0236–6290/95/$ 4.00 ©1995 *Akadémiai Kiadó, Budapest*

effects of hormones on *in vitro* maturation of goat follicular oocytes. Here we demonstrate that LH and estradiol stimulate the *in vitro* maturation of follicular oocytes in goat.

Materials and methods

Goat ovaries were collected from an abattoir and transported to the laboratory in normal saline at approximately 37 °C within 1 h.

Aspiration and in vitro culturing of follicular oocytes. The follicles (1–2 mm in diameter) were aspirated using a 20-gauge needle (4–6 oocytes recovered from each ovary). The oocytes (a total of 500) were divided into groups of 5–10 oocytes each and transferred in a 100 µl drop of medium TALP without epinephrine and taurine but containing Bovine Serum Albumin (BSA) (Tyrode's modified solution of Yanagimachi, 1982) and incubated in polypropylene petri dishes under liquid paraffin that was previously equilibrated with medium at 38.5 °C in 5% carbon dioxide/air mixture in a carbon dioxide incubator. Variable concentrations (5–20 µg/ml) of porcine LH (a gift from USDA, Animal Hormone Program, USA) and a fixed concentration (1 µg/ml) of estradiol (Sigma, USA) were injected into the culture drops containing oocytes under liquid paraffin. Incubation was carried out for 24 h at 38.5 °C. The morphological evaluation of oocytes at 0 h and 24 h were made under inverted microscope and the following observations were recorded: (*i*) cumulus expansion, (*ii*) germinal vesicle status and (*iii*) nuclear maturation. For the latter the oocytes were washed in 1% sodium citrate twice for 10 min each and then fixed in methanol : acetic acid (3:1) for 4–24 h. After fixation, the oocytes were transferred onto a clean glass slide and a cover slip with petroleum pads at the corner was gently lowered until a contact with the oocyte was made. The oocytes were stained with aceto-orcein and nuclear maturation was studied under oil immersion.

Results

The effects of variable concentrations of LH and a fixed concentration of estradiol on *in vitro* maturation of follicular oocytes in goat are summarized in Table 1. The sequence of the stages of oocyte maturation such as cumulus expansion, germinal vesicle breakdown (GVBD) and nuclear maturation (extrusion of polar body) were followed during incubation for 24 h. At 0 h of incubation all the oocytes were at the germinal vesicle stage and were enclosed in cumulus without any visible cytoplasmic contractions in them. During incubation, there was a progressive increase in cumulus expansion with mucilisation with increasing concen-

trations of LH and at a fixed concentration of estradiol. In the untreated control, at 0 h all the oocytes were at germinal vesicle stage and none of them reached the GVBD stage after 24 h of incubation. With increasing concentrations of LH (20 μg/ml) and at a fixed concentration of estradiol (1 μg/ml) 45% of the oocytes reached metaphase II (extrusion of polar body) stage of maturation and the remaining 55% were at metaphase I (see Table 1).

Table 1

The effect of LH and estradiol on the maturation percentage of follicular oocytes in goat

Treatments	Percentage of oocytes at			
	Germinal vesicle stage	Germinal vesicle breakdown	Metaphase	
			I	II
LH (5) + E	72.0	28.0	0	0
LH (10) + E	0	87.0	0	13.0
LH (20) + E	0	0	55.0	45.0

Numbers in parentheses indicate the concentration of LH in μg/ml. The concentration of estradiol in combination with LH was 1 μg/ml in each treatment. The period of incubation was 24 h.

Discussion

The results of the present study have demonstrated the beneficial effects of LH and estradiol on *in vitro* maturation of follicular oocytes in goat. This linear increase in oocyte maturation with rising concentrations of LH and at a fixed concentration of estradiol lends support to the role of these hormones in the maturation of follicular oocytes in goat. Similarly, the stimulatory effects of gonadotropins and steroids on *in vitro* maturation of oocytes have been reported in various species such as rat and mouse (Baker and Neal, 1972; Tsafriri et al., 1972, 1973; Eppig and Downs, 1988; Vanderhyden and Armstrong, 1989), rabbit (Thibault and Gerard, 1973; Bae and Foote, 1975), cow and pig (Fukui and Ono, 1989; Younis et al., 1989; Suss et al., 1990). In the present study we saw 45% stimulation of oocyte maturation at a 20 μg/ml concentration of LH and a fixed concentration of estradiol (1 μg/ml) in goat, whereas Moor and Trounson (1977) reported 51% stimulation of oocyte maturation with LH (1 μg/ml) in sheep. In the bovine this stimulation is reported to be 58–61.3% with LH (5 μg/ml) and FSH (2.5 μg/ml). When cultured *in vitro* in the presence of LH (20 μg/ml) and estra-

diol (1 μg/ml), hamster follicular oocytes that had previously been primed with PMSG and HCG showed 88% stimulation in maturation compared to the untreated control (Kaur et al., 1994).

Though the hormones stimulate *in vitro* maturation of follicular oocytes as shown here and also in other mammalian species, the mechanism by which these hormones stimulate oocyte maturation is still not very clear. Gonadotropins, particularly LH, might act through the granulosa cells, as in most of the previous studies as well as in the present work, it has been demonstrated that cumulus-enclosed oocytes show a better response to hormones than do the denuded ones. The luteinization of granulosa cells by LH, leading to the production of steroids, is well established and the presence of LH receptors on these cells has also been reported (Guraya, 1985). Some controversies, however, do exist regarding the role of steroids in oocyte maturation. Lindner et al. (1974) reported oocyte maturation in the presence of steroid inhibitors in the culture medium, but the developmental competence of these oocytes was not studied. Most of the other studies do show the beneficial effects of steroids in oocyte maturation, particularly in the acquisition of developmental competence (see Knobil et al., 1988), and the presence of steroid receptors on the nuclear membrane of oocyte has been reported. We feel that gonadotropins and steroids act synergistically in inducing proper oocyte maturation in goat, but their mechanism of action remains to be determined.

Acknowledgements

This study was funded by a grant to K.S.S. from Punjab State Council for Science of Technology, Chandigarh. The gift of LH from USDA Animal Hormone Program, USA is thankfully acknowledged.

References

Bae, I. H. and Foote, R. H. (1975): Effects of hormones on the maturation of rabbit oocytes recovered from follicles of various sizes. J. Reprod. Fertil. **42,** 357–360.

Baker, T. G. and Neal, P. (1972): Gonadotropin-induced maturation of mouse Graafian follicles in organ culture. In: Bigers, J. D. and Shultz, A. W. (eds) Oogenesis. University Park Press, Baltimore, pp. 377–396.

Bar Ami, S. and Tsafriri, A. (1981): Acquisition of meiotic competence in the rat: Role of gonadotropins and estrogens. Gamete Res. **4,** 453–472.

Chauhan, M.S. and Anand, S. R. (1991): *In vitro* maturation and fertilization of goat oocyte. Indian J. Exp. Biol. **29,** 105–110.

Cross, P. C. and Brinster, R. L. (1990): *In vitro* development of mouse oocytes. Biol. Reprod. **3,** 298–307.

Eppig, J. J. and Downs, S. M. (1988): Gonadotropin-induced oocyte maturation *in vivo* is not associated with decreased cyclic adenosine monophosphate in the oocyte cumulus cell complex. Gamete Res. **20,** 125–131.

Frylinck, M. D. and Kay G. W. (1992): Early developmental changes over time in *in vitro* matured *in vitro* fertilized ovine follicular oocytes. Proc. 12th Intl. Congr. Anim. Reprod. Vol. 2, pp. 841–843.

Fukui, Y. and Ono, R. (1989): Effects of sera, hormones and granulosa cells added to culture medium for *in vitro* maturation, fertilization, cleavages and development of bovine oocytes. J. Reprod. Fertil. **86,** 501–506.

Guraya, S. S. (1985): Biology of Follicles in Mammals. Springer-Verlag, Berlin – Heidelberg.

Karlach, V. (1986): The effect of FSH, LH, estradiol-17ß and progesterone on cytoplasmic maturation of bovine follicular oocytes *in vitro*. Folia Biol. (Praha) **33,** 259–265.

Kaur, J., Guraya, S. S. and Sidhu, K. S. (1994): Effect of LH and estradiol on *in vitro* maturation of hamster follicular oocytes. Indian J. Exp. Biol. **32,** 144–145.

Knobil, E., Neel, J. D., Ewing, L. L., Greenward, G. S., Market, C. L. and Pfaff, D. M. (1988): The Physiology of Reproduction. Vol. 1. Raven Press, New York.

Lindner, H. R., Tsafriri, A., Lieberman, M. E., Zor, U., Koch, Y., Bauminger, S. and Bornea, A. (1974): Gonadotropin action on cultured Graafian follicles: Induction of maturation division of the mammalian oocyte and differentiation of luteal cell. Rec. Prog. Horm. Res. **30,** 79–138.

Moor, R. M. (1978): Role of steroids in the maturation of ovine oocytes. Annls Biol. Anim. Biochim. Biophys. **18,** 477–482.

Moor, R. M. and Trounson, A. O. (1977): Hormonal and follicular factors affecting maturation of sheep oocytes *in vitro* and their subsequent developmental capacity. J. Reprod. Fertil. **40,** 101–109.

Pugh, P. A., Fukui, Y., Tervit, H. R. and Thompson, J. G. (1991): Developmental ability of *in vitro* matured sheep oocytes collected during nonbreeding season and *in vitro* fertilized with frozen ram semen. Theriogenology **36,** 771–778.

Smedt, V. de, Crozet, N., Ahmed-Ali, M., Martino, A., Cognie, V. and De-Smedt, V. (1992): *In vitro* maturation and fertilization of goat oocytes. Theriogenology **37,** 1049–1060.

Suss, U., Kassner, J., Wuthrich, K. and Stranzinger, G. (1990): Cumulus expansion, *in vitro* fertilization and embryo development after *in vitro* maturation of bovine oocytes in the presence of follicle stimulating or luteinizing hormones. Reprod. Domestic Anim. **25,** 3–13.

Thibault, C. and Gerard, M. (1973): Cytoplasmic and nuclear maturation of rabbit oocyte *in vitro*. Annls Biol. Anim. Biochim. Biophys. **18,** 477–482.

Tsafriri, A., Linder, N. R., Zor, U. and Lamprech, S. A. (1972): *In vitro* induction of meiotic division in follicle enclosed rat oocytes by LH, cyclic AMP and prostaglandin E_2. J. Reprod. Fertil. **31,** 39–50.

Tsafriri, A., Leiberman, M. E., Barnea, A., Mauminger, S. and Linder, H. R. (1973): Induction by luteinizing hormone of ovum maturation and steroidogenesis in isolated graffian follicles of the rat; Role of RNA and protein synthesis. Endocrinology **93,** 1378–1386.

Vanderhyden, B. C. and Armstrong, D. T. (1989): Role of cumulus cells and serum on an *in vitro* maturation, fertilization and subsequent development of rat oocytes. Biol. Reprod. **40,** 720–728.

Yanagimachi, R. (1982): Requirement of extracellular calcium ions for various stages of fertilization and fertilization related phenomena in the hamster. Gamete Res. **5,** 323–344.

Younis, A. I., Brackett, B. G. and Fayrer-Hoskin, R. A. (1989): Influence of serum and hormones on bovine oocyte maturation and fertilization *in vitro*. Gamete Res. **23,** 189–202.

Zhang, X. and Armstrong, D. T. (1989): Effect of follicle stimulating hormone and ovarian steroids during fertilization of rat oocyte. Gamete Res. **23,** 267–278.

Zuelka, K. A. and Brackett, B. G. (1990): Luteinizing hormone enhanced *in vitro* maturation of bovine oocytes with and without protein supplementation. Biol. Reprod. **43,** 784–787.

Acta Veterinaria Hungarica 43 (1), pp. 95–103 (1995)

THE PRODUCTION OF K88 ANTIGEN BY *Escherichia coli* AND *Salmonella typhimurium* STRAINS WITH RECOMBINANT DNA

E. HOLODA and I. MIKULA

Department of Microbiology and Immunology, University of Veterinary Medicine, Komenského 73, 041 81 Košice, Slovak Republic

(Received February 25, 1994)

Expression of K88ab antigen by strains with recombinant DNA, differing in molecular weight or promoter, was subjected to investigation. Strains with recombinant DNA produced greater amounts of antigen than did field isolates. Maximal production was recorded for the *E. coli* C600 strain with 10.85 kb recombinant DNA with promoter P1 of the pBR322 vector. The substitution of promoter P_{tac} for promoter P1 did not result in an increased expression of K88ab antigen. The production of K88ab antigen by *Salmonella typhimurium* TM333 with recombinant DNA was on a level comparable to that of *E. coli* C600 or *E. coli* HB101 strains with the same recombinant DNA.

Key words: *E. coli, Salmonella typhimurium,* production, K88 antigen, recombinant DNA, P_{tac} promoter, P1 promoter, induction

The K88 antigen is one of the adhesins which take part in the pathogenesis of *E. coli* diarrhoea in newborn piglets (Ørskov et al., 1964). Its expression is affected by physical and chemical factors of the environment (Nagy et al., 1977). Stabilisation of the expression is important from the commercial point of view. Maximum production of K88 antigen is achieved by the production of strains with recombinant DNA (Mooi et al., 1986; Aitken and Hirst, 1993). These types of strains are used for the preparation of pilus vaccines (Gyles and Maas, 1987; Attridge et al., 1988; Hofmann and Schweder, 1993).

Studies of the stimulation of local immunity use attenuated *Salmonella* strains as "carriers" of plasmids which encode heterologous virulence determinants, e.g. those from *E. coli* (Pardon et al., 1986; Brown et al., 1987; Curtiss/III et al., 1988).

In both cases it is vitally important to select the most efficient possible DNA-recombinant K88 producers. Furthermore, it also seemed to be important to determine the dynamics and inducibility of the K88.

0236–6290/95/$ 4.00 ©1995 *Akadémiai Kiadó, Budapest*

Materials and methods

Bacterial strains and cultivation

The bacterial strains used are listed in Table 1. Recipient bacterial strains and *E. coli* 7301 were cultivated either in Minca or in LB medium at 37 °C with intensive shaking.

Table 1

Characterisation of bacterial strains

Ser. no.	Strain	Characteristics	Presence of plasmids
1	*E. coli* HB101	Recipient strain	No plasmids
2	*E. coli* C600	Recipient strain	No plasmids
3	*E. coli* MT	Recipient strain (lac i^Q host), gift from Sedláček et al. (1986)	No plasmids
4	*S. typhimurium*	Recipient strain, gift from Pardon et al. (1986)	No plasmids
5	*E. coli* 7301	Field isolate $K88^+$ (from piglets with diarrhoea)	Polyplasmid
6	*E. coli* 68/I	gift from Guinée and Jansen (1979) $K88^+$	Polyplasmid
7	*E. coli* e37	*E. coli* HB101 $K88^+$	74 kb plasmid DNA from (5)
8	*E. coli* e376	*E. coli* HB101 $K88^+$	11.6 kb *Hind* III-*Hind* III from (7) in *Hind* III site pBR322 (promoter P1 from pBR 322)
9	*E. coli* M1	*E. coli* HB101 $K88^+$	10.85 kb from (8) obtained by excision of a 5.15 kb *Eco* RI-*Eco* RI partial fragment (promoter P1 from pBR 322)
10	*E. coli* C1	*E. coli* C600 $K88^+$	10.85 kb from (9)
11	*E. coli* M1 P_{tac}	*E. coli* MT $K88^+$	10.85 kb from (9), where promoter P1 was replaced with promoter P_{tac} (from pKK 223-3)
12	*S. typhim.* $K88^+$	*S. typhimurium* $K88^+$	10.85 kb DNA from (9) transformed into (4)

Note: Recombinant DNA was constructed according to Holoda and Mikula (1994)

E. coli production strains with recombinant K88 DNA (e376, M1, C1 and *S. typhimurium* $K88^+$) were cultivated in Minca and LB media with ampicillin (100 $\mu g \times ml^{-1}$) at 37 °C with intensive shaking.

The *E. coli* M1 P_{tac} strain was cultivated at 37 °C in Minca medium without glucose. The P_{tac} promoter was induced with IPTG (isopropyl beta – D – thiogalactoside, Pharmacia) or with lactose.

Isolation of K88ab antigen and preparation of K88ab antiserum

Quantitative determination was carried out after the thermal isolation of K88ab antigen. A sediment of bacteria cultured overnight, obtained by centrifugation, was resuspended in a 1/25 part of the original volume using phosphate buffer of pH 7.2, warmed up to 65 °C for 15 min and centrifuged. The K88ab antigen was determined in the supernatant by the passive haemagglutination method. The molecular weight of the K88ab antigen was determined using SDS–PAGE (Laemmli, 1970).

The K88ab antiserum was prepared by isolation of antigen from *E. coli* 68/I strain by gel filtration (Mooi and De Graaf, 1980), homogenisation with a complete Freund's adjuvant in the concentration 1 $mg \times ml^{-1}$, using a ratio of 2:1, and applied s.c. (1.5 ml) at five locations to rabbits of the Vienna white breed with an average liveweight of 2.5 kg. Immunisation was repeated 5 times at 7-day intervals. Rabbits were bled 14 days after the last immunisation. The resulting titer of K88ab antibodies, determined by passive haemagglutination (PHA), was 1:4,096. The serum was stored at –20 °C. The purity of the used immunogen was verified by SDS-PAGE.

Quantitative determination of K88ab antigen by the PHA method

Freeze–dried ram erythrocytes from TK HEM ÚSOL–Prague were used in the PHA. The following reaction conditions were considered optimal: pH 7.2; sensitisation of erythrocytes with K88 antigen for 1 h at 37 °C; sensitisation with an antiserum for 1 h at 56 °C, or for 2 h at 37 °C; 1:1 (V/V) ratio of erythrocytes and antigen (antiserum) for sensitisation; 2–fold resultant concentration of sensitised erythrocytes (in relation to the initial volume); 1 % normal rabbit serum was used for washing the erythrocytes after sensitisation as well as for use as a diluent for PHA.

Results

Investigation of the production of K88ab antigen by strains *E. coli* e37, e376, M1, C1 and *Salmonella typhimurium* TM333 $K88^+$ revealed that maximum expression of antigen is achieved after 18 h of cultivation. Study of the

time dynamics of expression of K88ab antigen in strains *E. coli* M1, C1 and *Salmonella typhimurium* TM333 K88$^+$ showed maximum expression of K88ab antigen in the second half of the exponential phase of growth (Fig. 3). The exponential phase of growth was recorded between hours 2 and 5 in the LB medium and between hours 3 and 6 in the Minca medium with 0.15 % of glucose.

Table 2 indicates that the *E. coli* C1 strain is the best producer of K88ab antigen. The PHA reaction aimed at determining the K88ab antigen isolated from the above strain after 5 h of cultivation in the LB medium was positive up to a dilution of 1:2,048, while after 18 h of cultivation it provided positive results up to a dilution of 1:4096.

Table 2

Comparison of expression of K88 antigen in *Escherichia coli* and *Salmonella typhimurium* strains

Ser. no.	Strain	PHA reaction at dilution of isolated K88 antigen*			
		After 5 h		After 18 h	
		Minca	LB	Minca	LB
1	*E. coli* HB 101	N	N	N	N
2	*E. coli* C600	N	N	N	N
3	*E. coli* MT	N	N	N	N
4	*S. typhimurium*	N	N	N	N
5	*E. coli* 7301	1:4	1:8	1:16	1:16
6	*E. coli* e37	–	–	1:8	1:32
7	*E. coli* e376	–	–	1:256	1:512
8	*E. coli* M1	1:512	1:1024	1:1024	1:2048
9	*E. coli* C1	1:1024	1:2048	1:1024	1:4096
10	*E. coli* M1 P_{tac}	1) N	–	1) N	–
		2) 1:2048	–	2) 1:2048	–
		3) 1:2048	–	3) 1:4096	–
11	*S. typhimurium* K88$^+$	1:258	1:1024	1:512	1:2048

Legend: The starting sample was diluted 1:4 to eliminate nonspecifities = K; PHA = passive haemagglutination; N = undetected; 1 = without induction; 2 = induced with IPTG; 3 = induced with lactose; – = untested

The reaction of K88ab antigen obtained from a field isolate *E. coli* 7301 after 5 h cultivation was positive up to the dilution 1:8 and after 18 h of cultivation up to the dilution 1:16. The production of K88ab antigen by *E. coli* M1 strain is comparable with that of *E. coli* C1 (Fig. 1).

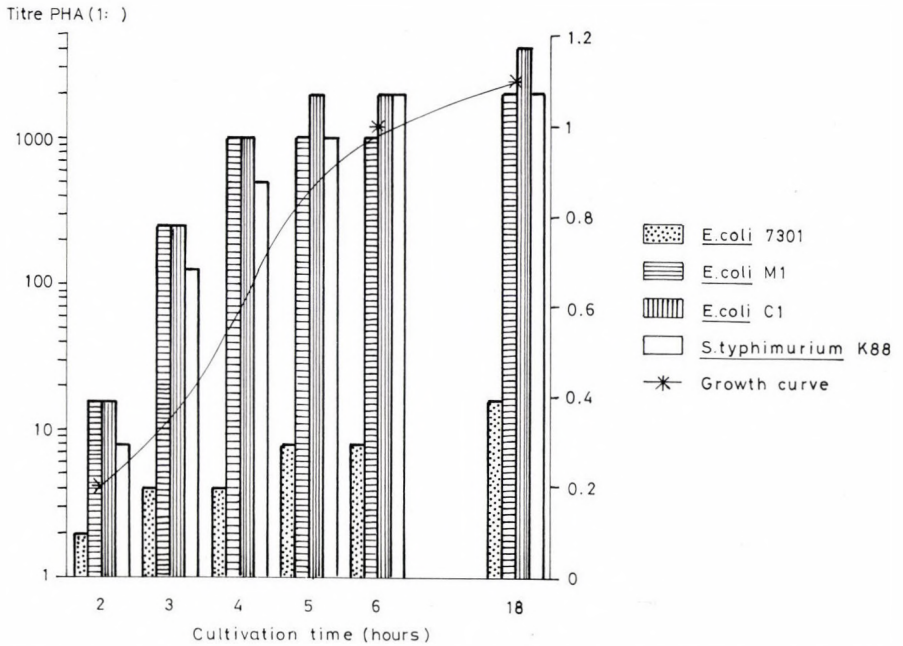


Fig. 1. Production of K88 antigen by *E. coli* M1, C1 (recDNA-P1 promoter), 7301 (field isolate) and *S. typhimurium* TM333 K88$^+$ (recDNA-P_{tac} promoter) strains — LB medium

Expression of *E. coli* M1 with P_{tac} promoter after induction with lactose in the Minca medium was comparable with that of *E. coli* 8:21:261 in the LB medium after 18 h of cultivation (Table 2). Despite a negative PHA reaction obtained for *E. coli* M1 P_{tac} strain when cultivated without an inductor (Table 2), a line in the zone of K88ab antigen was observed in the lysate of bacterial cells analysed by means of SDS-PAGE.

Testing of expression of K88ab antigen with *E. coli* M1 P_{tac} strain by means of PHA showed that a lactose concentration equal to 40 mM $\times$ l^{-1} is optimal for the induction of P_{tac} promoter, while a 30 mM $\times$ l^{-1} concentration is optimal for IPTG (Fig. 2). Further increase of lactose concentration fails to increase the level of expressed K88 antigen. On the contrary, raising the concentration of IPTG to 50 mM $\times$ l^{-1} decreased the level of expressed K88 antigen. The time of adding lactose up to the 3rd hour of cultivation did not exert a significant effect on the expression of K88ab antigen (Fig. 3).

Recombinant DNA, transformed from *E. coli* M1 into *Salmonella typhimurium* TM333 strain, showed long-term stability. Expression of K88ab antigen by *Salmonella typhimurium* TM333 K88$^+$ strain was on the level shown by

the *E. coli* M1 strain (Fig. 1). The properties of K88ab antigen produced by field isolates or strains with recombinant DNA were analogous.

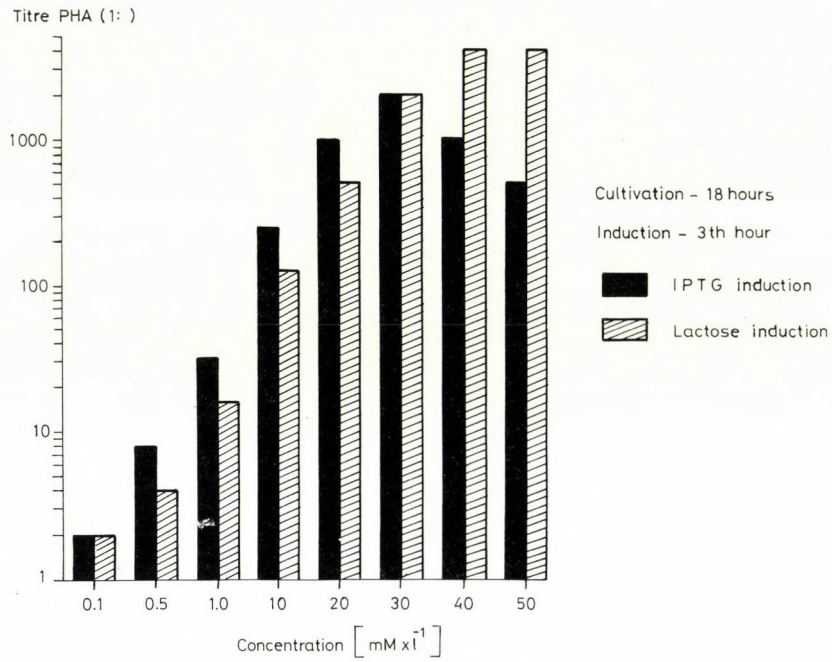


Fig. 2. Dependence of K88 antigen production by *E. coli* M1 P_{tac} (recDNA-P_{tac} promoter) strain on IPTG and lactose concentration, respectively (IPTG and lactose were added in the 3rd hour of cultivation) — Minca medium

Discussion

Isolation of fimbriae as immunogens for the production of vaccines remains a topical problem. Bacterial strains with recombinant DNA and a suitable arrangement of transcription and translation mechanisms are capable of expressing, under defined conditions, sufficient quantities of the required immunogen (Brown et al., 1986; Scott, 1984).

Numerous biological, chemical and physical factors affect expression of proteins regulated by the DNA (Hofmann and Schweder, 1993). Isaacson (1985) stated that passage of field *E. coli* K88 isolates has a negative influence on the expression of fimbrial antigens. He explains this by the formation of lipopolysaccharide envelopes or by the loss of plasmid DNA. This disadvantage may be eliminated in strains with recombinant DNA by the prolonged storage of recombi-

MAGYAR
TUDOMÁNYOS AKADÉMIA
KÖNYVTÁRA

nant DNA in a frozen state in buffer or in alcohol. Such DNA may be transformed, when needed, into a recipient strain.

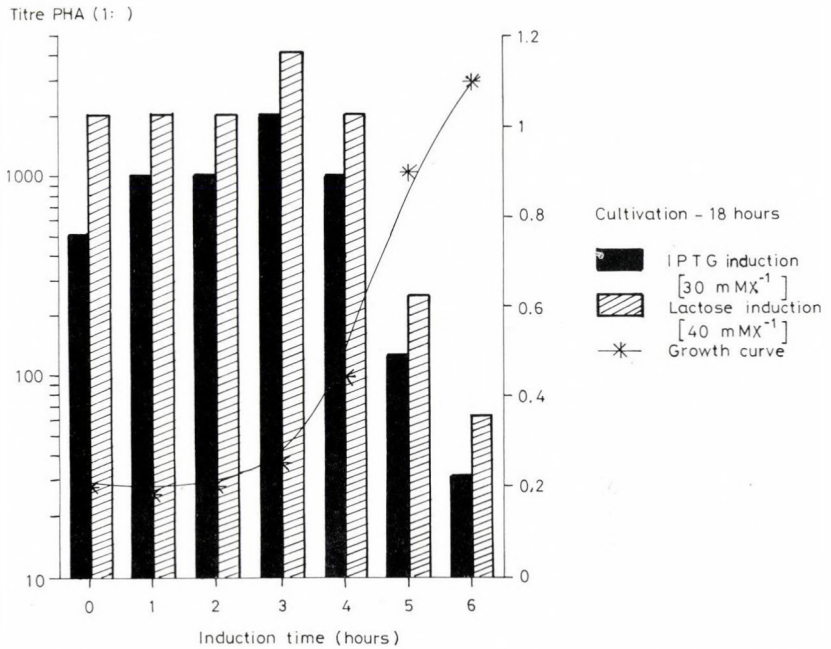


Fig. 3. Dependence of K88 antigen production by *E. coli* M1 P_{tac} (recDNA-P_{tac} promoter) on the induction time of the promoter with IPTG (30 mM × 1^{-1}) and with lactose, respectively (40 mM × 1^{-1}) — Minca medium

The results of studies on expression of K88ab antigen encoded by recombinant DNAs indicate that the expression of K88ab antigen is positively affected by the decrease of size of the DNA fragment down to 6.4 kb and the insertion of a strong P1 promoter of the vector pBR322. Increased number of copies of recombinant DNA in a cell (Stüber and Bujard, 1981) was obtained by cloning into the multicopy vector pBR322, which also increased the expression of the required antigen. Stabilisation of recombinant DNA in a host cell was achieved by addition of ampicillin to the cultivation medium (the gene of resistance to ampicillin in recombinant DNA comes from the pBR322 vector).

The expression of required proteins encoded by recombinant DNA may be positively affected by means of the so-called "strong" promoters (Scott, 1984; Hofman and Schweder, 1993). Our studies were carried out using promoter P_{tac} inducible with IPTG or lactose. In the strain designated *E. coli* M1P_{tac} we failed to achieve an increase in the expression of K88ab antigen in comparison with strains *E. coli* M1 or *E. coli* C1 in which the promoter P1 controlled the expres-

sion of K88 antigen. The results also indicated that lactose was a better inducer than IPTG. When *E. coli* $M1P_{tac}$ was cultivated in the Minca medium with ampicillin and without inducers (lactose, IPTG), thermal processing by the use of the PHA reaction failed to detect K88ab antigen. However, using SDS–PAGE, a pronounced line in the zone of K88 antigen was detected in the bacterial cell lysate. This may suggest that the K88 antigen is not transported across the cell membrane to the bacterial cell surface.

The effort to replace the promoter P1 with promoter P_L (obtained from temperature-activated lambda phage) did not result in the expression of K88ab antigen by suitable recipient strains despite the fact that the presumed structure of recombinant DNA was achieved.

Attenuated *Salmonella* strains were also used as carriers of heterogeneous genetic information (Clements, 1987; Kaper and Levine, 1988). Our studies on the expression of K88ab antigen encoded by recombinant DNA (from *E. coli* M1) revealed that *S. typhimurium* TM333 strain presents a suitable recipient for expression of the gene encoding the K88 antigen. This genetic information in *S. typhimurium* TM333 was retained even after 50 passages in a medium containing ampicillin. Expression of the K88 antigen with *Salmonella typhimurium* TM333 strain after 5 h of cultivation in LB medium reached a value of 1:2,046 in the PHA reaction (Fig. 1). A short-term heating resulted in the release of K88ab antigen from the surface of *S. typhimurium* TM333 $K88^+$ strain. SDS-PAGE was used to confirm the molecular weight of the isolated immunogen.

The results point to the possibility of using *E. coli* C1, M1, $M1P_{tac}$ and *Salmonella typhimurium* TM333 strains with recombinant DNA encoding K88ab antigen for experimental vaccination.

References

Aitken, R. and Hirst, T.R. (1993): Recombinant enterotoxins as vaccines against *Escherichia* coli-mediated diarrhoea. Vaccine **11,** 227–233.

Attridge, S., Hackett, J., Morona, R. and Whyte, P. (1988): Towards a live oral vaccine against enterotoxigenic *Escherichia coli* of swine. Vaccine **6,** 387–390.

Brown, F., Chanok, R. M. and Lerner, R. A. (1986): Vaccines 86: New Approaches to Immunization. Cold Spring Harbor Laboratory, 1986.

Brown, A., Hormaeche, C. E., Demarco de Hormaeche, R., Winther, M., Dougan, G., Maskell, D. J., Stocker, B. A. D. (1987): An attenuated aro A *Salmonella typhimurium* vaccine elicited humoral and cellular immunity to cloned–galactosidase in mice. J. Infect. Dis. **155,** 86–92.

Clements, J. D. (1987): Use of attenuated mutants of *Salmonella* as carriers for delivery of heterologous antigens to the secretory immune system. Pathol. Immunopathol. Res. **6,** 137–146.

Curtiss III, R., Goldschmidt, R. M., Fletchall, N. B. and Kelly, S. M. (1988): Avirulent *Salmonella typhimurium* cys crp oral vaccine strains expressing a streptoccocal colonization and virulence antigen. Vaccine **6,** 155–160.

Guinée, P. A. M. and Jansen, H. W. (1979): Behaviour of *E. coli* K antigens K88ab, K88ac and K88ad in immunoelectrophoresis double diffusion and hemagglutination. Infect. Immun. **23,** 700–705.

Gyles, C. L. and Maas, W. K. (1987): Recombinant DNA technology and enterotoxigenic *E. coli* vaccines. Prog. Vet. Microbiol. Immunol. **3,** 139–158.

Hofmann, K. H. and Schweder, T. (1993): *Escherichia coli* host/plasmid systems providing biological containment. In: Wöhrmann, K. and Tomiuk, J. (eds) Transgenic Organisms. Birkhäuser Verlag, Basel, Switzerland, 1993. pp. 193–208.

Holoda, E. and Mikula, I. (1994): The construction of recombinant K88 DNA with P_{tac} promoter. Folia Microbiol. (in press).

Isaacson, R. E. (1985): Development of vaccines for bacterial diseases using recombinant DNA technology. Avian Dis. **30,** 28–35.

Kaper, J. B., Levine, M. M. (1988): Progress towards a vaccine against enterotoxigenic *E. coli.* Vaccine **6,** 197–199.

Laemmli, U. K. (1970); Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227,** 680–685.

Mooi, F. R. and De Graaf, F. K. (1980): Isolation and characterization of K88 angitens. FEMS Microbiol. Lett. **45,** 17–20.

Mooi, F. R., Claassen, I., Bakker, D., Kuipers, H. and De Graaf, F. K. (1986): Regulation and structure of an *E. coli* gene coding for an outer membrane protein involved in export K88 ab fimbrial subunits. Nucleic Acids Res. **14,** 2443–2457.

Nagy, B., Moon, H. W. and Isaacson, R. E. (1977): Colonization of porcine intestine by enterotoxigenic *E. coli*: Selection of piliated forms *in vivo*, adhesion of piliated forms to epithelial cells *in vitro* and incidence of a pilus antigen among porcine enteropathogenic *E. coli*. Infect. Immun. 16, 344–352.

Ørskov, I., Ørskov, F., Sojka, W. J. and Witting, W. (1964): K antigens K88ab (L) and K88ac (L) in *E. coli*. A new O antigen: O147 and A new K antigen: K99 (B). Acta Pathol. Microb. Scand. Sect. **B,** 439–447.

Pardon, P., Popoff, M. Y., Coynault, C., Marly, J., Miras, I. (1986): Virulence-associated plasmids of *Salmonella* serotype *typhimurium* in experimental murine infection. Ann. Inst. Pasteur/Microbiol. (Elsevier) **137D,** 47–60.

Sedláček, J., Fábry, M., Kašpar, P., Zadražil, S. and Kaprálek, F., (1986): Plasmid construct for overproduction of chymosin in *E. coli*. Biotechnology and Bioindustry **6,** 17–19.

Scott, J. R. (1984): Regulation of plasmid replication. Microbiol. Rev. **48,** 1–23.

Stüber, D. and Bujard, H. (1984): Organization of transcriptional signals in plasmids pBR322 and pACYC184. Proc. Natl. Acad. Sci. USA **78,** 167–171.

Acta Veterinaria Hungarica 43 (1), pp. 105–115 (1995)

INTESTINAL ABSORPTION OF COLOSTRAL LYMPHOCYTES IN NEWBORN LAMBS AND THEIR ROLE IN THE DEVELOPMENT OF IMMUNE STATUS

S. TUBOLY[1], S. BERNÁTH[2], R. GLÁVITS[3], Andrea KOVÁCS[2] and Z. MEGYERI[1]

[1]Department of Microbiology and Infectious Diseases, University of Veterinary Science, H–1581 Budapest, P.O. Box 22; [2]Control Institute for Veterinary Vaccines, Pharmaceuticals and Feed, Budapest, Hungary; [3]Central Veterinary Institute, Budapest, Hungary

Two model experiments were conducted to study the intestinal absorption of colostral lymphoid cells and the role of these cells in the development of immune status in newborn lambs. In experiment I, 17 lambs of 14 Merino ewes were used. Suspensions of lymphoid cells separated from the colostrum (cell density: 5×10^6/ml) and blood (3×10^6/ml) were labelled with technetium ($Na^{99m}TcO_4$) of 37 MBq/ml radioactive concentration. In three groups of lambs, 10-ml volumes of the cell suspensions were injected directly into the duodenum after laparotomy, while in a fourth group (group Ia) the same volume was administered to the animals through an oesophageal tube. The labelled cells revealed that colostral cells of the lamb's own dam are absorbed from the gastrointestinal tract and get into the newborn lamb's lymph circulation irrespective of the route of application. In experiment II, involving 40 lambs of 40 ewes, we studied the effect of absorbed colostral lymphocytes on the development of the newborn lamb's immune status. Twenty ewes (group A) each were treated with 3 ml tetanus anatoxin twice, while the remaining animals (group B) were left uninoculated. Lambs of group A (designated A2) were separated from their dams immediately after birth, then were administered, through an oesophageal tube, 10 ml of a suspension of lymphoid cells (cell density: 5×10^6/ml) separated from the maternal colostrum. Subsequently, the lambs were interchanged with lambs of nonimmunized ewes of group B (designated lambs B1), i.e. were mutually put out to nursing. At three days of age, lambs of groups A1, A2, B1 and B2 were inoculated with 3 ml tetanus anatoxin, then blood samples were taken from them 5 times in a period of 27 days for comparative examination of the humoral and cellular immune reactions. The results demonstrate that lymphoid cells from the colostrum of the lambs' own dam become absorbed into the newborn lambs' lymph circulation, remain immunologically active and may transfer, besides immunological memory, also cellular activity.

Key words: Colostral lymphocytes, intestinal absorption, newborn lamb, immune status

0236–6290/95/$ 4.00 ©1995 *Akadémiai Kiadó, Budapest*

In species with an epitheliochorial placenta, colostrum-derived maternal immunoglobulins play a decisive role in the passive protection of newborn animals (Porter et al., 1970; McDowell, 1973; Bourne and Curtis, 1973; Brown et al., 1975; Newby and Bourne, 1977; Butler et al., 1981; Banks 1982; Newby et al., 1982). At the end of pregnancy, numerous lymphoid cells from the mother's "common mucosal system" (Bienenstock, 1974; Solmon, 1987) also accumulate in the mammary gland. These cells include T and B lymphocytes (Parmely and Beer, 1977; Roux et al., 1977; Manning and Parmely, 1980; Seelig, 1980) which are transferred into the newborn animal's digestive tract with the colostrum.

There is a diversity of opinion concerning the role of these cells. According to some authors, they exert a protective effect in the digestive tract, while others suggest that through their mediator substances they may result in partial tolerance and graft-versus-host reaction (Beer et al., 1975; Head et al., 1977). In rats and sheep, Sheldrake and Husband (1985) as well as Seelig and Head (1987) demonstrated that syngeneic and allogeneic maternal peripheral lymphocytes may absorb from the digestive tract. The intestinal absorption of colostral lymphocytes in swine was demonstrated by us earlier (Tuboly et al., 1988).

This study consisted of two model experiments. In the first experiment the absorption of lymphocytes from syngeneic and allogeneic maternal colostrum was studied. The aim of the second experiment was to determine whether the absorbing cells can transfer immunological information of any kind to the newborn animals.

Materials and methods

Model experiment I: Study of the absorption of colostral cells

Experimental animals. Seventeen newborn lambs of 14 Merino ewes were used. The lambs were separated from their mothers immediately after birth and treated according to the experimental design shown in Table 1.

Separation of lymphoid cells. After the lambs' birth, 50-ml blood samples were taken from the ewes into heparinized tubes, and 100-ml colostrum samples were collected from them after the intravenous injection of 2 ml oxytocin (10 IU/ml).

Lymphoid cells were separated from the blood samples with Ficoll-Paque, the suspension was washed in PBS and adjusted to a cell density of 3×10^6/ml.

Colostrum samples were centrifuged at 500 rpm for 40 min at 4 °C. After removing the lipid layer, the cell sediment was resuspended in PBS, washed twice and the density of the cell suspension made with PBS was adjusted to 5×10^6/ml after having checked the ratio of live cells by trypan blue staining (85–90%).

Labelling of lymphoid cells. The cells were labelled with isotonic NaCl solution (100 MBq/ml) of $Na^{99m}TcO_4$ (Barth and Gillespie, 1974).

Table 1

Design of experiment I

Group	No. of fetus	Colostral cells from syngeneic dams	Colostral cells from allogeneic dams	Blood lymphocytes from syngeneic dams	Route of application
	1a				
	2				
I	3	10 ml	–	–	laparotomy
	4	5×10^6			(duodenum)
	5a				
	6				
	7a				
Ia	8	10 ml	–	–	oesophagus
	9	5×10^6			
	1b				
II	5b	–	10 ml		laparotomy
	7b		5×10^6		(duodenum)
	10				
III	11	–	–	10 ml	laparotomy
	12			3×10^6	(duodenum)
	13				laparotomy
IV	14	–	–	–	(duodenum)
					10 ml PBS

Explanation: lambs designated 1a, 5b and 7b (group II) are twins of lambs designated 1a, 5a and 7a (groups I and Ia), respectively

To the lymphoid cell suspension 37 MBq/ml $Na^{99m}TcO_4$ was added and incubated for 15 min at 37 °C. Subsequently, ^{99m}Tc was reduced by adding 0.04 ml/ml sterile 0.2% $SnCl_2 \times 2\ H_2O$ freshly prepared in acidic citrate dextrose solution, then the cell suspensions were incubated for further 15 min.

Technetium not bound to the cells was removed from the suspensions by four cycles of centrifugation in PBS solution. After that, the cells were resuspended in an amount of PBS solution corresponding to the original volume.

After labelling and washing, the suspensions were checked for the ratio of cell-bound and free radioactivity with an NZ-310 Autogamma Spectrometer (Gamma Works, Budapest). After the washing of labelled cells 96–98% of the radioactivity was cell bound.

Treatment of lambs. The labelled cells were administered to the lambs 8–10 hours after birth, either by directly injecting them into the duodenum after laparot-

omy (groups I, II and III) or by administering them via an oesophageal tube (group Ia).

Nine lambs (groups I and Ia) were treated with syngeneic colostral cells, 3 lambs (group II) received colostral cells from allogeneic ewes, while further 3 lambs (group III) were treated with lymphoid cells derived from the blood of syngeneic ewes.

Two lambs of group IV were used as control and received PBS solution free from cells by injection into the duodenum following laparotomy.

In the 8th hour after treatment with the labelled cells, the lambs were exsanguinated in ketamin-HCl anaesthesia (16 mg/kg body mass), and samples were taken from the duodenum, jejunum, mesenteric lymph ducts and mesenteric lymph nodes. Some of the samples were embedded by the method of Sainte-Marie (1962) while from the remaining part cryostat sections were prepared directly. The sections were examined by autoradiography after coating with Ilford-Nuclear-G emulsion, incubation in dark room at 4 °C for 6 days, fixation after development in ORWO A-49 solution, staining with haemalum and evaluation in light microscope.

Histological examinations. Samples taken from the duodenal and jejunal segments of the digestive tract and from the mesenteric lymph nodes were fixed in formaldehyde solution buffered with NaH_2PO_4 and NaOH (pH 7.2), embedded in paraffin, sectioned, and the sections were stained with P. Mayer's acid haemalum and eosin.

Electron microscopy. Samples taken from the jejunum were fixed in 2.5% glutaraldehyde buffered with sodium cacodylate, then in 1% osmium tetroxide. After embedding in Durcupan resin, so-called semi-thin sections of approx. 1 μm thickness were prepared, stained with toluidine blue and evaluated by light microscopy. Ultra-thin sections were counterstained with uranyl acetate and lead citrate and then examined in a Philips 201 CS electron microscope.

Model experiment II: Study of the effect of colostral cells on the development of immune status

Experimental animals. Forty Merino ewes and their 40 lambs were included in the experiment. The ewes were fertilized simultaneously after oestrus synchronization.

According to the experimental design (Table 2), 20 ewes (group A) were vaccinated twice with tetanus anatoxin (Phylaxia, Budapest) at an interval of 3 months during pregnancy (the second vaccination was carried out 3 weeks before term). The remaining 20 ewes (group B) were left unvaccinated.

Table 2

Design of experiment II

Ewes	Group	A			B
	No.	20			20
	Vaccine	Tetanus anatoxin			–
Lambs	Group	A1	A2 ⇄	B1	B2
	No.	10	10	10	10
	Colostral calls	–	5×10^6/ml	–	–
	Vaccine	Tetanus anatoxin			
	Sampling	days 0, 3, 6, 10, 17 and 27			

Of the lambs of immunized ewes, group A1 lambs remained with their dams. Lambs of group A2 were weaned immediately after birth and given, through an oesophageal tube, 10 ml of a suspension of lymphoid cells (5×10^6 cells/ml) separated from the colostrum of syngeneic dams, then put out to nursing to nonimmunized ewes belonging to group B. The lambs of these latter ewes (B1) were transferred to the dams of lambs A2, whereas lambs of group B2 remained with their own dams.

Subsequently, at 3 days of age the lambs were inoculated with tetanus anatoxin. Blood samples were taken from them before vaccination and then on a total of 5 occasions up to 27 days of age.

The tetanus antibody titre of the sera was measured by RIA (Bernáth and Habermann, 1974). The sensitivity of the method was 0.004 IU/ml.

The *LST indices* were determined as described previously (Tuboly et al., 1987) by adding 10 IU of tetanus anatoxin to lymphoid cell cultures suspended in Leighton tubes. The ratio of blastogenesis was determined by autoradiography on the basis of ^{3}HTdR incorporation.

Results

Model experiment I

In cryostat sections prepared from lambs of *groups I and Ia* (treated with lymphoid cells from syngeneic maternal colostrum) the labelled cells were demonstrable by autoradiography in the epithelium of duodenal and jejunal samples (Fig. 1), in the content of mesenteric lymph ducts (Fig. 2) and in the cortical zone of the mesenteric lymph nodes.

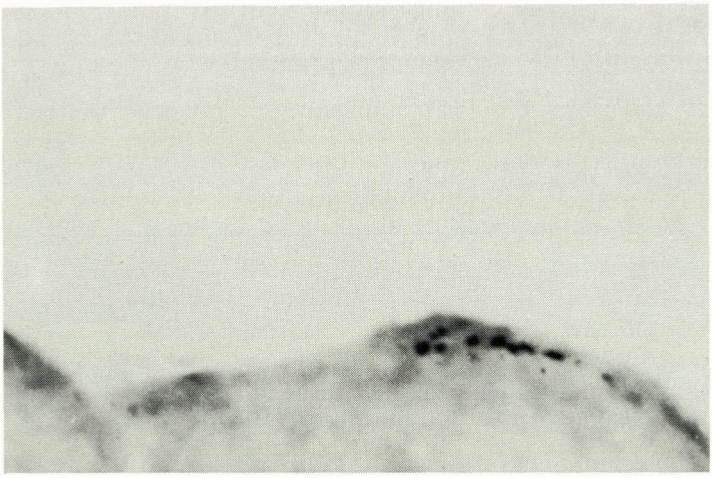


Fig. 1. Labelled cells in the duodenal epithelium. Cryostat section, × 120

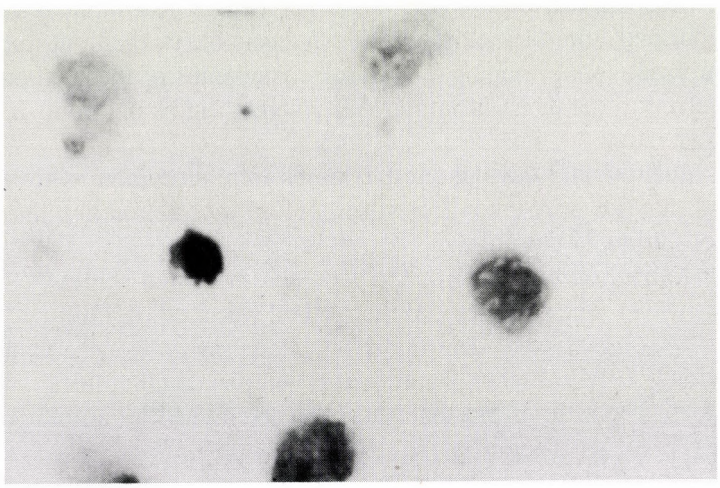


Fig. 2. Labelled cell in the content of the mesenteric lymph duct. × 260

By histological and electron microscopic examination of sections prepared from the duodenal and jejunal samples, the labelled lymphoid cells were demonstrable in the lumen, at the apical part of intestinal villi and among epithelial cells of the jejunal mucosa, in the dilated intercellular spaces (Fig. 3).

In *group II* (lambs treated with lymphoid cells from allogeneic maternal colostrum), large numbers of labelled cells could be recognized in the lumen of the intestinal segments examined. However, these cells had not become absorbed, as

they could not be demonstrated in the mucosal epithelium of duodenal and jejunal samples, in the lymph and in the lymph nodes.

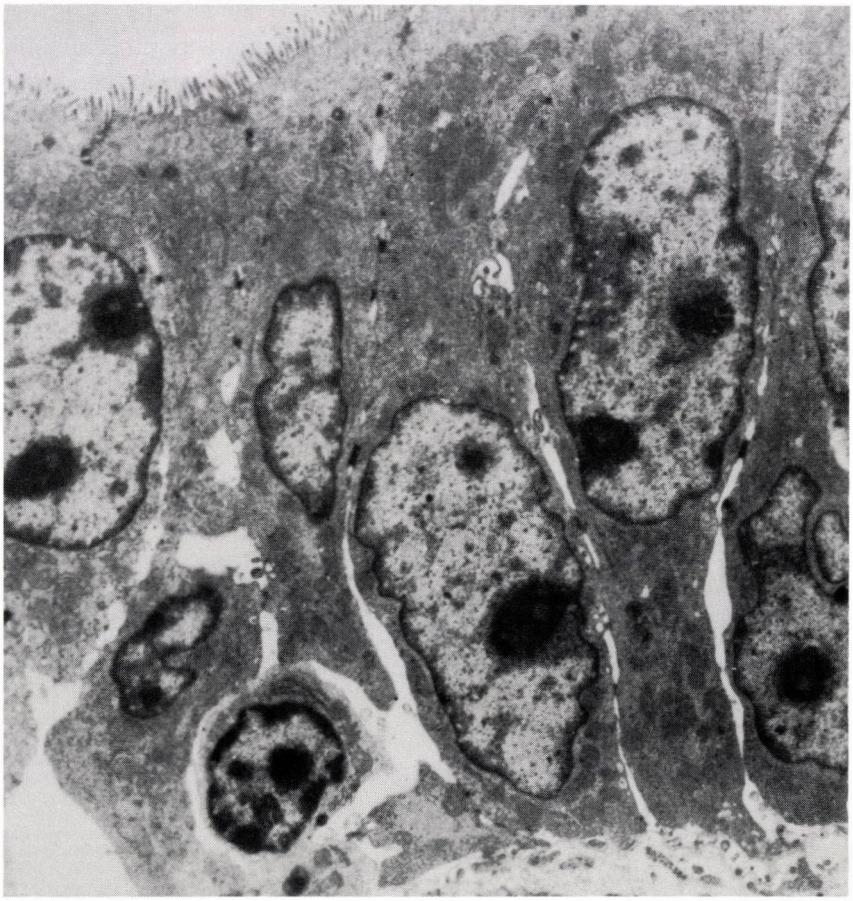


Fig. 3. Lymphocyte in the intercellular space among the jejunal epithelial cells. Electron micrograph, × 9,600

In lambs of *group III*, lymphoid cells derived from the maternal blood could be observed in the epithelium of duodenal and jejunal samples but were absent from both the lymph and the mesenteric lymph nodes.

Duodenal, jejunal and mesenteric lymph node samples of the *control animals* showed a normal histological picture corresponding to age.

Model experiment II

The kinetics of humoral immune response in lambs of *groups A2 and B2* is illustrated in Fig. 4. On postvaccination days 17–21, lambs which had received colostral cells from their own immunized dams (A2) had higher titres of antibodies (0.038 IU/ml) than did the control (B2) animals. Peripheral lymphocytes from lambs of group A2 responded to *in vitro* antigenic stimulation with 12–15% blastogenic transformation, whereas in lambs of group B2 the ratio of transformed cells was 4–5%.

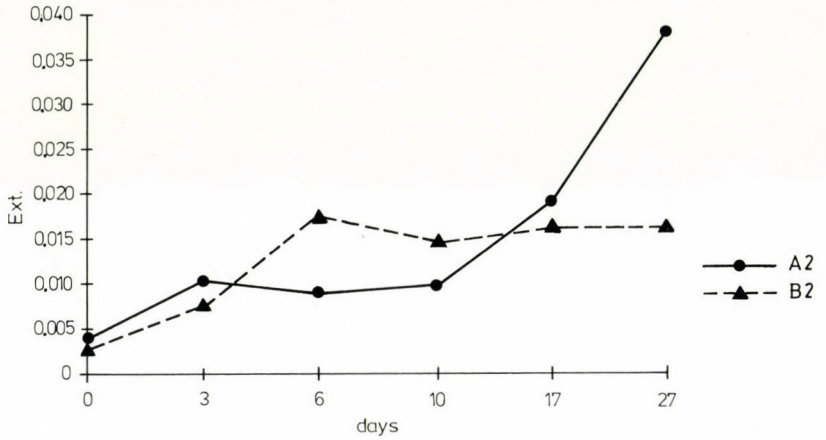


Fig. 4. Kinetics of humoral immune response in lambs of groups A2 and B2

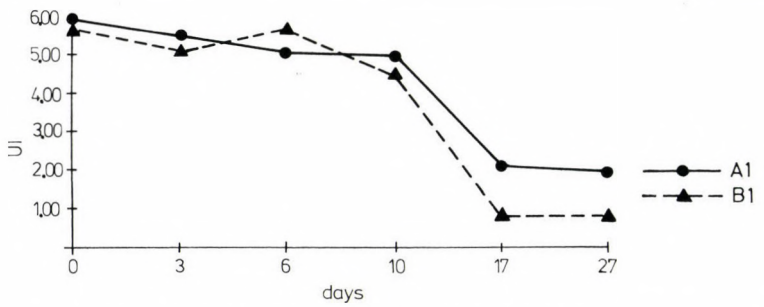


Fig. 5. Antibody titres in lambs of groups A1 and B1 (solid line: group A1; broken line: group B1)

The results obtained for groups A1 and B1 are shown in Fig. 5. It appears that maternal immunoglobulins taken up with the colostrum inhibited the development of the lambs' active immune response through a "feedback" effect. By LST, a 3–4% rate of blastogenic transformation was found for samples from these animals.

Discussion

The ovine colostrum contains approx. 10^6 lymphoid cells per ml. The majority of these cells are polymorphonuclear cells (41–84%) and macrophages (8–49%), while the ratio of lymphocytes is 6–11% (Lee and Outteridge, 1981).

Lymphoid cells are derived from the "common mucosal system", accumulate in the mammary gland (Parmely and Beer, 1977), then get transepithelially into the alveoli (Seelig and Beer, 1978; Seelig, 1980) from where they are transferred into the newborn's digestive tract via the colostrum.

In these experiments, it was found that cells from syngeneic maternal colostrum become absorbed from the digestive tract and reach the mesenteric lymph nodes by the 8th hour after their uptake.

This study has demonstrated that cells from allogeneic maternal colostrum fail to cross the gastrointestinal barrier, presumably due to MHC incompatibility. Though lymphoid cells derived from the blood of syngeneic mothers could be observed in the mucosal epithelium, they failed to reach the mesenteric lymph nodes. These observations suggest that the absorption of colostral lymphoid cells may be regulated by mechanisms similar to those controlling migration into, and cumulation in, the mammary gland as well as transepithelial transfer (Seelig and Beer, 1987; Manning and Parmely, 1980).

Our results support the view of Kmetz et al. (1970), Seelig and Billingham (1981) and Weiler et al. (1983) that lymphoid cells can cross the gastrointestinal barrier. In contrast, other authors (Silvers and Poole, 1975; Miller, 1981) could not observe such absorption, perhaps due to inadequate sensitivity of the methods applied. In this study, colostral cells were labelled with the radioisotope technetium 99. The advantages of technetium 99 labelling in immunological tests were demonstrated by Barth et al. (1974). The primary advantage is that it does not affect the cells' protein and nucleic acid metabolism to a detectable extent, spontaneous radioisotope release is low, and the free isotope does not bind to the cells again.

Several possibilities have been suggested regarding the method of cell absorption. According to Sheldrake and Husband (1985), cells may get into the epithelial cells through the microvilli in the same way as immunoglobulin molecules. Others (Ogra et al., 1977; Seelig and Billingham, 1981) believe that absorption may take place through the intercellular spaces of the epithelial layer. In earlier studies on the absorption of immunoglobulins (Széky et al., 1979) we found that immunoglobulins get into the cytoplasm of intestinal epithelial cells by endocytosis, after "pushing apart" the microvilli. In contrast, our electron microscopic findings indicate that the absorption of lymphoid cells may take place through the intercellular spaces of the epithelial layer.

Model experiment II has revealed that in lambs treated with colostral cells from immunized ewes (group A2) antibodies appeared sooner (on days 17–21

after vaccination) and in higher titres (0.038 IU/ml) than in group B2 (0.016 IU/ml). This allows us to conclude that in group A2 cells carrying immunological memory were transferred with the colostral lymphocytes, resulting in a secondary type immune response to tetanus anatoxin. On the other hand, in group B2 the appearance of antibodies followed the kinetics of primary immune response. This is supported by the observation that in lambs of group A2 the LST indices elicited by specific antigenic stimulation exceeded those of the control lambs by 8–12% on the average. The antigenic commitment of colostral lymphocytes has already been demonstrated in a model experiment performed with Aujeszky's disease virus in swine (Tuboly et al., 1987).

The results of this study indicate that lymphocytes from syngeneic maternal colostrum reach the newborn lamb's lymph circulation, remain immunologically active and may transfer immune memory; thus, they may play a role in the development of the newborn's immune status.

References

Banks, K. L. (1982): Host defense in the newborn animal. J. Am. Vet. Med. Assoc. **181,** 1053–1056.

Barth, R. F. and Gillespie, G. Y. (1974): The use of technetium99m as a radioisotopic label to assess cell-mediated immunity *in vitro*. Cell. Immunol. **10,** 38–49.

Beer, A. E., Billingham, R. E. and Haad, J. R. (1975): Natural transplantation during suckling. Transplant. Proc. **7,** 399–404.

Bernáth, S. and Habermann, E. (1974): Solid phase radioimmunoassay in antibody coated tubes for the quantitative determination of tetanus antibodies. Med. Microbiol. Immunol. **160,** 47–51.

Bienenstock, J. (1974): The physiology of the local immune response and the gastrointestinal tract. In: Brent, L. and Halborow, J. (eds) Progr. Immunol. Vol. II. 4. North Holland Publishing, p. 197.

Bourne, F. J. and Curtis, J. (1973): The transfer of immunoglobulin IgG, IgA and IgM from serum to colostrum and milk in the sow. Immunology **24,** 157–162.

Brown, P. J., Bourne, F. J. and Denny, H. R. (1975): Immunoglobulin containing cells in the pig mammary gland. J. Anat. **120,** 329–335.

Butler, J. E., Klobasa, F. and Werham, E. (1981): The differential localisation of IgA, IgM and IgG in the gut of suckled geonatal piglets. Vet. Immunol. Immunopathol. **2,** 53–65.

Head, J. R., Beer, A. E. and Billingham, R. E. (1977): Significance of the cellular component of the maternal immunologic endowment in milk. Transplant. Proc. **9,** 1465–1471.

Kmetz, M., Dunne, H. W. and Schultz, R. D. (1970): Lekocytes as carriers in the transmission of bovine leukemia. Am. J. Vet. Res. **31,** 637–641.

Lee, C. S. and Outteridge, P. M. (1981): Leucoc]ytes of sheep colostrum, milk and involution secretion, with particular reference to ultrastructure and lymphocytes subpopulations. J. Dairy Res. **48,** 225–237.

Manning, L. S. and Parmely, M. J. (1980): Cellular determinants of mammary cell mediated immunity in the rat. I. The migration on radioisopropically labelled T lymphocytes. J. Immunol. **125,** 2508–2514.

McDowell, G. H. (1973): Local antigenic stimulation of guinea pig mammary gland. Aust. J. Exp. Biol. Med. Sci. **51,** 237–245.

Miller, S. C. (1981): Failure to demonstrate morphologically the presence of colostral or milk cells in the wall of the gastrointestinal tract of the suckling neonatal mouse. J. Reprod. Immunol. **3,** 187–194.

Newby, T. J. and Bourne, F. J. (1977): The nature of the local immune system of the bovine mammary gland. J. Immunol. **118,** 461–465.

Newby, T. J., Stockes, C. R. and Bourne, F. J. (1982): Immunological activities of milk. Vet. Immunol. Immunopathol. **3,** 67–94.

Ogra, S. S., Weintraub, D. and Ogra, P. L. (1977): Immunologic aspects of human colostrum milk. III. Fate and absorption of cellular and soluble components in the gastrointestinal tract of newborn. J. Immunol. **119,** 245–248.

Parmely, M. J. and Beer, A. E. (1977): Colostral cell mediated immunity and the concept of common secretory immune system. J. Dairy Sci. **60,** 655–661.

Porter, P., Nookes, D. E. and Allen, D. W. (1970): Secretory IgA and antibodies to *Escherichia coli* porcine colostrum and milk and their significance in the alimentary canal of the young pig. Immunology **18,** 245–257.

Roux, M. E., McWilliams, M., Phillips Quagliata, J. M., Weisz Carrington, M. and Lamm, M. E. (1977): Origin of IgA secreting plasma cells in mammary gland. J. Exp. Med. **146,** 1311–1332.

Sainte Marie, G. (1962): A paraffin embedding technique for studies employing immunofluorescence. J. Histochem. **10,** 250–256.

Seelig, L. L. Jr. (1980): Dynamics of leucocytes in rat mammary epithelium during pregnancy and lactation. Biol. Reprod. **22,** 1211–1217.

Seelig, L. L. Jr. and Beer, A. E. (1978): Transepithelial migration of leucocytes in mammary gland of lactating rats. Biol. Reprod. **18,** 736–741.

Seelig, L. L. and Billingham, R. E. (1981): Capacity of "transplanted" lymphocytes to traverse in intestinal epithelium of adult rats. Transplantation **32,** 208–314.

Seelig, L. L. and Head, J. R. (1987): Uptake of lymphocytes fed to suckling rats. An autoradiographic study of the transit of labeled cells through the neonatal gastric mucosa. J. Reprod. Immunol. **10,** 258–297.

Sheldrake, R. F. and Husband, A. J. (1985): Intestinal uptake of intact maternal lymphocytes by neonatal rats and lambs. Res. Vet. Sci. **39,** 10–15.

Silvers, W. K. and Poole, T. W. (1975): The influence of foster nursing on the survival and immunologic competence of mice and rats. J. Immunol. **115,** 1117–1121.

Solmon, H. (1987): The intestinal and mammary immune system in pigs. Vet. Immunol. Immunopathol. **17,** 367–388.

Széky, A., Rátz, F., Tuboly, S. and Nagy, Gy. (1979): Absorption of colostral immunoglobulins in suckling piglets. Acta Microbiol. Acad. Sci. Hung. **26,** 99–110.

Tuboly, S., Medveczky, I. and Bálint, A. (1987): Lymphoid cells of porcine colostrum (in Hungarian, with English abstract). Magyar Állatorvosok Lapja **42,** 613–616.

Tuboly, S., Bernáth, S., Glávits, R. and Medveczky, I. (1988): Intestinal absorption of colostral lymphoid cells in newborn piglets. Vet. Immunol. Immunopathol. **20,** 75–87.

Weiler, I. J., Hickler, W. and Sprenger, R. (1983): Demonstration that milk cells invade the suckling neonatal mouse. Am. J. Reprod. Immunol. **4,** 95–98.

Acta Veterinaria Hungarica 43 (1), pp. 117–124 (1995)

CONTAMINATION OF BROILER CHICKEN'S MASH AND LITTER WITH MOULDS, AFLATOXINS, OCHRATOXIN A AND ZEARALENONE

M. ŠKRINJAR[1], M. RISTIĆ[1] and Z. GRBIĆ[2]

[1]Faculty of Technology, University of Novi Sad, Bulevar Cara Lazara 1, 21000 Novi Sad, Yugoslavia; [2]Poultry Slaughterhouse DP "Yuko", 2300 Žitište, Yugoslavia

(Received March 20, 1994)

Contamination of broiler chicken's mash and litter with moulds, aflatoxin B1 (AB1), G1 (AG1), ochratoxin A (OA) and zearalenone (F-2 toxin) was examined on a broiler fattening farm. All broiler chicken's mash samples tested were contaminated with moulds. The highest total viable counts of moulds (3.3×10^5 per g) were found in broiler starter diet. Moulds isolated from mash were classified into 8 genera and 27 species. Many of them (*Aspergillus* spp., *Fusarium* spp., *Penicillium* spp.) are known as mycotoxin-producing moulds. *Fusarium moniliforme* was the dominant species in mash mycopopulations. Moulds were also found in all litter samples. The highest fungus count (80×10^5 per g) in litter was found at the beginning of fattening. Fungi isolated from litter samples belonged to 9 genera and 22 species. *Aspergillus, Penicillium* and *Mucor* were the most prevalent genera. None of the broiler chicken's mash and litter samples tested was contaminated either with AB1, AG1, OA or with F-2 toxin. *F. moniliforme* was zearalenone-negative also under laboratory conditions.

Key words: Moulds, aflatoxins, ochratoxin A, zearalenone, broiler chicken, mash, litter

Moulds and their toxic metabolites are known among the most important pollutants of broiler chicken feed mixtures (Romo and Fernandez, 1986; Heperkan and Alperden, 1988). Severe mould contamination of the mash impairs its hygienic quality and makes it unsuitable for fattening purposes.

Toxigenic moulds are often present in feed mixture mycopopulations. These fungi can produce various mycotoxins, which are ingested by the animal with the contaminated feed and can eventually be detected in the meat.

With a view to the high prevalence of fungi in the environment including livestock enterprises, the aim of this study was to investigate the distribution of moulds, especially toxigenic ones, and the possible presence of some mycotoxins in broiler chicken's mash and litter during the fattening.

0236–6290/95/$ 4.00 ©1995 *Akadémiai Kiadó, Budapest*

Materials and methods

The contamination of broiler chicken's mash and litter with moulds, aflatoxin B1 (AB1), G1 (AG1), ochratoxin A (OA) and zearalenone (F-2 toxin) was examined.

The investigations were carried out during a 7-week period in the autumn of 1992 at a broiler fattening unit in Vojvodina. In that period the enterprise housed 17,972 one-day-old "Hibro" broiler chickens.

Wheat straw was used as litter. Before the broilers were stocked in, the litter was disinfected with formalin.

The microclimatic conditions of the broiler enterprise were as follow: at the beginning of fattening the ambient temperature was between 30 and 33 °C. Subsequently the temperature decreased by 1 to 2 °C per week and at the end of fattening it was 18 °C. The relative humidity of air was 60 to 80%.

In the first 20 days broiler chickens were fattened with starter, in the subsequent 21 days with finisher I and during the last 8 days with finisher II.

The composition of the broiler chicken's mash is given in Table 1.

Table 1

Composition of broiler chicken's mash (in %)

Ingredient	Starter	Finisher I	Finisher II
Corn	33.57	37.80	35.92
Wheat	20	20	15
Soybean meal	16.91	18.14	21.97
Fodder meal	10	10	15
Fish meal	8.5	5	3
Soybean oil meal	–[a]	4.97	2.75
Dicalcium phosphate	1.71	1.96	2.19
Premix	1	1	1
Calcium carbonate	0.81	0.71	0.75
Sodium chloride	0.25	0.25	0.30
Methionine	0.03	0.12	0.12
Stimulant	0.05	0.05	–
Sunflower seed 44%	5	–	2.05
Soybean oil	0.16	–	–
Fodder yeast	2	–	–

[a] not added

At the end of this experiment the broiler chickens' average body mass was 2,075 g. Feed conversion was 2.52 kg, the daily body mass gain was 37.52 g and the average mortality rate was 9.08%.

Feed mixture and litter samples for mycological and mycotoxicological analyses were taken immediately before feeding on day 1, 11, 21, 32, 42 and 49 of fattening as average samples.

Mycological analyses. The dilution plate technique was used for isolating moulds from feed and litter samples and for determining their total viable counts per g. Before dilution preparation, the litter samples were ground aseptically. Then 20 g of the sample were diluted in 180 ml sodium chloride solution and homogenized for 10 min in homogenizer. Petri dishes (in triplicates) were inoculated with 1 ml of each dilution (10^{-1}, 10^{-2}, 10^{-3} and 10^{-4}). Incubation was carried out at 25 °C for 5, 7 and 10 days. Sabouraud dextrose agar with streptomycin (0.01–0.02%) was used as isolation medium. After the incubation, colonies with different morphological characteristics were inoculated on appropriate media.

Identification of mould species was done according to Ellis (1971), Pidopličko and Miljko (1971), Nelson et al. (1983), Samson and van Reenen-Hoekstra (1988) and Samson et al. (1976).

Mycotoxicological analyses. All test samples were analyzed simultaneously for the presence of AB1, AG1, OA and F-2 toxin. Qualitative and quantitative determination of these mycotoxins was performed using a TLC method according to the Official Methods of Analysis of the A.O.A.C. (1990).

Since *Fusarium moniliforme* was found to be the most prevalent mould in mycopopulations isolated from feed samples, its ability to produce F-2 toxin under laboratory conditions was examined in a further experiment.

For that purpose, sterilized wheat kernels (50 g) were inoculated with 10 ml of *F. moniliforme* inoculum. Incubation was carried out at 26 to 28 °C for 14 days. On days 2 and 3 of incubation 10 ml of distilled water was added to each Erlenmayer flask (300 ml). Extraction of F-2 toxin and its determination were done as mentioned above.

Results and discussion

Mycological analyses. Table 2 shows that all samples were contaminated with moulds. The highest total viable count of moulds (3.3×10^5 per g) was found in broiler chicken's starter (sample 2). The fact, that mouldy starter was used for the fattening at the early stages of the experiments, when they were still weak, has probably effected their daily growth decrease and mortality rate.

Prolonged incubation time of inoculated Petri dishes, from five to ten days, had not significantly effected the increase of total viable counts of moulds in feed mixture.

Table 2

Total viable counts of moulds per g ($\times 10^8$) of broiler chicken's mash

Sampling day	Sample no.	Incubation time		
		Day 5	Day 7	Day 10
1	1	1.1	1.4	1.4
11	2	3.3	3.3	3.3
21	3	0.2	0.2	0.2
32	4	0.9	1.2	1.2
42	5	1.1	1.1	1.5
49	6	0.7	0.7	0.7

1, 2 – broiler chicken starter; 3, 4, 5 – finisher I; 6 – finisher II

According to the Yugoslav regulations (S1. list SFRJ, br. 2, 1990), mixed feed with mould counts exceeding 5×10^4 per g is hygienically unsatisfactory for feeding young animals. Having this in mind, in these experiments only one sample (no. 3) was of appropriate mycological quality.

Some other authors have also reported on high fungal contamination of mixed feed. Benham (1981) reported that in 11 out of 136 samples of poultry mixed feed, the fungal counts were higher than 10^5 per g. Investigating the mycological quality of mixed feed, Heperkan and Alperden (1988) found that 82 samples were contaminated with moulds at a very high degree (10^4–10^6/g).

The moulds isolated from broiler chicken's mash were classified into 8 genera and 27 species as follows: *Alternaria alternata* (Fr.) Keissler, *A. tenuissima* (Kunze ex Pers.) Wiltshire, *Aspergillus flavus* Link, *A. fumigatus* Fresen., *A. niger* van Tiegh., *A. repens* (Cda.) De Bary, *A. sydowi* (Bain. and Sart.) Thom and Church, *A. terreus* Thom, *Botrytis cinerea* Pers. ex Pers., *Cladosporium cladosporioides* (Fr.) de Vries, *C. herbarum* (Pers.) Link ex Gray, *Fusarium lateritium* Nees, *F. moniliforme* Sheldon, *F. solani* (Mart.) Sacc., *F. sporotrichioides* Sherb., *Mucor dimorphosporus* Lendn., *M. hiemalis*Wehmer, *M. petrinsularis* Naumov, *M. plumbeus* Bon., *M. racemosus* Fres., *M. sinensis* Miljko et Beljakova, *Penicillium aurantiogriseum* Dierckx, *P. brevi-compactum* Dierckx, *P. claviforme* Bain., *P. commune* Thom, *P. frequentans* Westling, *P. variabile* Sopp and *Scopulariopsis brevicaulis* (Sacc.) Bainier.

Many of these species, especially those belonging to the genera *Aspergillus, Fusarium* and *Penicillium*, are toxigenic and can produce various toxic metabolites (Marasas et al., 1984; Samson and van Reenen-Hoekstra, 1988; Mills, 1990). These genera were found to have a significant share in mycopopulations isolated from the feed mixture in these experiments (Fig. 1). It is also necessary to point out the dominant presence of *Fusarium moniliforme*, a potential producer of

zearalenone and some other toxic metabolites (Marasas et al., 1984), in the feed samples tested.

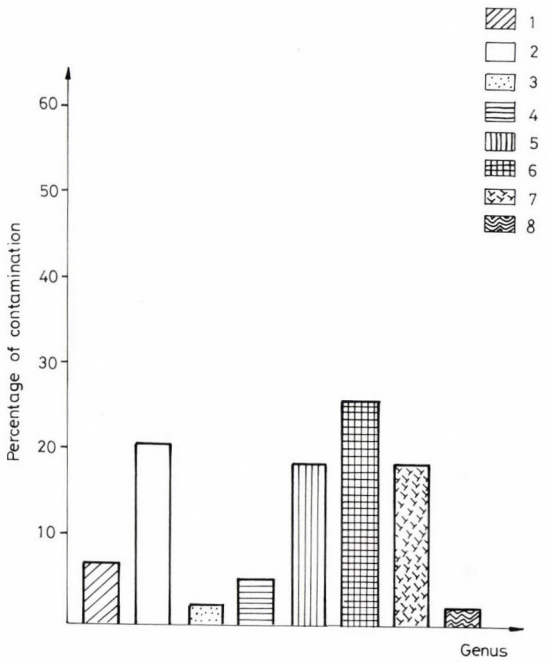


Fig. 1. Occurrence of fungal genera in broiler chicken's mash. 1: *Alternaria* spp.; 2: *Aspergillus* spp.; 3: *Botrytis* sp.; 4: *Cladosporium* spp.; 5: *Fusarium* spp.; 6: *Mucor* spp.; 7: *Penicillium* spp.; 8: *Scopulariopsis* spp.

F. moniliforme was isolated from starter (samples 1 and 2) and finisher I (samples 3 and 5). Having in mind the share of cereals (about 58%, Table 1) in the composition of broiler chicken's feed mixtures, it was assumed that the cereals were the source of feed contamination with *Fusarium* species.

At the beginning of fattening, feed and water were poured into the mash hopper and water control equipment manually. As a result, much feed and water were spilled and the litter became moist. Such litter, with admixture of spilled feed, was a good substrate for fungal growth. The highest total viable count of moulds per g (80×10^5) was found at that time (Table 3). At a later stage, when the chickens got accustomed to using a water fountain and feed-box by themselves, less water was spilled and the litter dried. These new conditions resulted in a drastic decrease in the total viable counts of fungi.

Table 3

Total viable counts of moulds per g ($\times 10^5$) of litter

Sampling day	Sample no.	Incubation time		
		Day 5	Day 7	Day 10
1	L1	27	27	29
11	L2	77	77	80
21	L3	41	41	41
32	L4	13	20	20
42	L5	0.6	0.6	0.6
49	L6	0.2	0.2	0.3

Numerous fungal strains were isolated from the litter. They were classified into 9 genera and 22 species: *Absidia ramosa* (Lindt) Lendn., *Aspergillus candidus* Link, *A. clavatus* Desm., *A flavus* Link, *A. fumigatus* Fresen., *A. repens* (Cda.) De Bary, *A. terreus* Thom, *A. versicolor* (Vuill.) Tiraboschi, *Fusarium moniliforme* Sheldon, *Geotrichum candidum* Link ex Pers., *Mucor hiemalis* Wehmer, *M. petrinsularis* Naumov, *M. racemosus* Fres., *M. sinensis* Miljko et Beljakova, *Penicillium aurantiogriseum* Dierckx, *P. chrysogenum* Thom, *P. frequentans* Westling, *P. granulatum* Bainier, *P. spinulosum* Thom, *Rhizopus nigricans* Ehrenb., *Scopulariopsis brevicaulis* (Sacc.) Bainier and *Syncephalastrum racemosum* Cohn ex Schroet.

By the number of species isolated, *Aspergillus, Penicillium* and *Mucor* were found to be the most important genera. They were present with 7, 5 and 4 species, respectively. The genus *Fusarium* was represented by one species only, *F. moniliforme*.

The distribution of fungal genera in litter is given in Fig. 2.

Mycotoxicological analyses. None of the broiler chicken's mash and litter samples tested was contaminated with the mycotoxins AB1, AG1, OA and zearalenone.

Since *F. moniliforme* was commonly occurred in feed mixture samples not contaminated with zearalenone, the ability of *F. moniliforme* to produce zearalenone was tested under laboratory conditions.

It was established that *F. moniliforme*, which was isolated from feed samples in these experiments, was zearalenone-negative also under laboratory conditions. This result can be explained by the lack of genetic properties necessary for zearalenone synthesis.

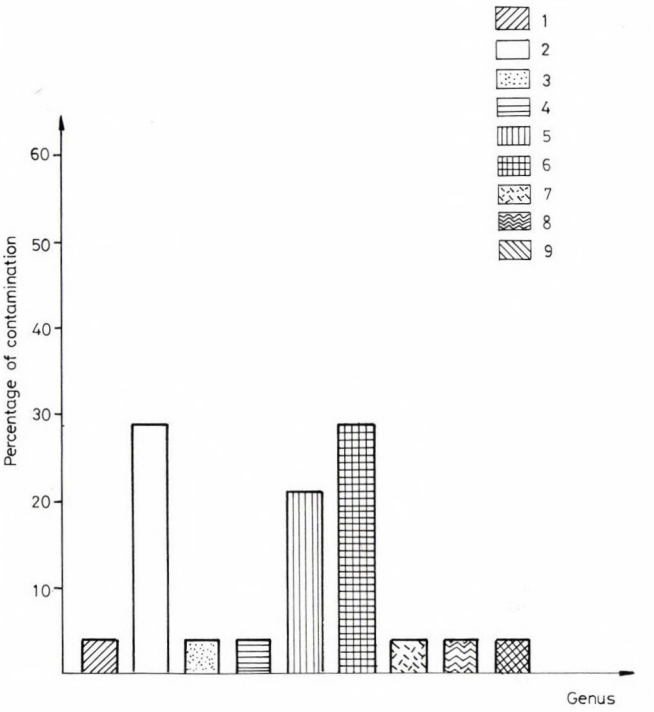


Fig. 2. Occurrence of fungal genera in litter. 1: *Absidia* sp.; 2: *Aspergillus* spp.; 3: *Fusarium* sp.; 4: *Geotrichum* sp.; 5: *Mucor* spp.; 6: *Penicillium* spp.; 7: *Rhizopus* sp.; 8: *Scopulariopsis* sp.; 9: *Syncephalastrum* sp.

References

Benham, C. L. (1981): Moulds and mycotoxins in animal feeding stuffs 1980. Proceedings of the 4th Meeting on Mycotoxins and Animal Disease, Weybridge, 1–3 April 1981, pp. 38–40.

Ellis, M. B. (1971): Dematiaceous Hyphomycetes. Commonwealth Mycological Institute, Kew and Surrey, England, 507 pp.

Heperkan, D. and Alperden, I. (1988): Mycological survey of chicken feed and some feed ingredients in Turkey. J. Food Protection **51,** 807–810.

Marasas, W. F. O., Nelson, P. E. and Toussoun, T. A. (1984): Toxigenic *Fusarium* species. Identity and Mycotoxicology. The Pennsylvania State University Press, University Park and London, xix + 328 pp.

Mills, J. T. (1990): Mycotoxins and toxigenic fungi on cereal grains in western Canada. Can. J. Physiol. Pharmacol. **68,** 982–986.

Nelson, P. E., Toussoun, T. A. and Marasas, W. F. O. (1983): *Furasium* species. An Illustrated Manual for Identification. The Pennsylvania State University Press, University Park and London, ix + 193 pp.

Official Methods of Analysis of the Association of Official Analytical Chemist (1990): Natural Poisons (Scott, P. M. ed.), pp. 1184–1213.

Pidopličko, N. M. and Miljko, A. A. (1971): Atlas mukoraljnih gribov. Izdateljstvo "Naukova dumka", Kiev, 115 pp.

Romo, M. A. M. and Fernandez, G. S. (1986): Aflatoxin-producing potential of *Aspergillus flavus* strains isolated from Spanish poultry feeds. Mycopathologia **95,** 129–132.

Samson, R. A. and van Reenen-Hoekstra, E. S. (1988): Introduction to Food-borne Fungi. Centraalbureau voor Schimmelcultures, Baarn, 3rd ec., 299 pp.

Samson, R. A., Stolk, A. C. and Hadlok, R. (1976): Revision of the subsection Fasciculata of *Penicillium* and some allied species. Studies in Mycology, no. 11. Centraalbureau voor Schimmelcultures, Baarn, 47 pp.

S1. list SFRJ (1990): Rule book on maximal allowed quantities of harmful substances in feed. No. 2, pp. 27–31.

Acta Veterinaria Hungarica 43 (1), pp. 125–137 (1995)

HISTOLOGICAL CHANGES IN THE SWIMBLADDER WALL OF EELS DUE TO ABNORMAL LOCATION OF ADULTS AND SECOND STAGE LARVAE OF *Anguillicola crassus*

K. MOLNÁR[1], J. SZAKOLCZAI[2] and F. VETÉSI[2]

[1]Veterinary Medical Research Institute, Hungarian Academy of Sciences, H–1581 Budapest, P.O. Box 18, Hungary; [2]Department of Pathology, University of Veterinary Science, H–1400 Budapest, P.O. Box 2, Hungary

(Received May 20, 1994)

In severe *Anguillicola crassus* infection of eels, adult helminths and 2nd stage larvae staying in the swimbladder lumen may occasionally get, through minor lesions of the tunica interna, into the subserosa of the swimbladder wall where they die and disintegrate. A thin connective tissue capsule is formed around the helminths that behave as foreign bodies in intercellular location, while the lacunas of the surrounding loose connective tissue comprise melanin-containing macrophages. In the environment of the 2nd stage larvae the formation of giant cells is a typical finding.

Key words: *Anguillicola crassus,* Nematoda, eel, swimbladder, abnormal location, histopathology

Since *Anguillicola crassus* was introduced into Europe in the mid-1980s, (Neumann, 1985) numerous papers have dealt with its prevalence (Peters and Hartmann, 1986; Taraschewski et al., 1987; Hartmann, 1987; Dupont and Petter, 1988; Belpaire et al., 1989; Koops and Hartmann, 1989; Kennedy and Fitch, 1990; Koie, 1991; Székely et al., 1991; Moravec, 1992), life cycle (De Charleroy et al., 1990; Haenen and van Banning, 1991; Höglund and Thomas, 1992; Thomas and Ollevier, 1992), seasonal occurrence (van Willigen and Dekker, 1989), and pathogenic effect exerted on the host (Mellergaard, 1988; Haenen et al., 1989; Boon et al., 1989; Boon et al., 1990*a,b,c*; van Banning and Haenen, 1990; Molnár et al., 1991; Möller et al., 1991; Sprengel and Lüchtenberg, 1991; Höglund et al., 1992). At the same time, data on the histopathological changes caused by the parasite can be found only in the works of Haenen et al. (1989), van Banning and Haenen (1990), and Molnár et al. (1993). The latter authors gave a detailed description of the general lesions produced by adult worms and by larvae in the mucous membrane of the swimbladder and in the subserosa, but only incidentally mentioned the mechanical injury caused to the swimbladder wall. At the same time, Liewes and Schaminee-Main (1987) and Kamstra (1990) graded by severity the swimbladder lesions caused by anguillicolosis in eels. They regarded

0236–6290/95/$ 4.00 © 1995 *Akadémiai Kiadó, Budapest*

as the most severe lesion the rupture of the swimbladder wall and the appearance of a brownish-blackish substance consisting of nematode debris in the swimbladder wall that had been replaced by a thick layer of connective tissue.

This paper presents the histopathological changes resulting from the anguillicolosis-induced discontinuity of the swimbladder wall, and provides data on the histogenesis of the granuloma-like lesions caused by helminths and larvae abnormally entering the swimbladder wall, as well as on the nature of the pigmentation which is seen in some cases.

Materials and methods

The material used in this study comprised eels that had been derived from Lake Balaton and dissected in 1992 (Molnár et al., 1993). In 1993, complementary studies were carried out: of 344 eels caught from different regions of Lake Balaton, only those specimens which exhibited changes resulting from swimbladder lesions (helminths and 2nd stage larvae located in the swimbladder wall, or intensive pigment formation) were processed for histology. In contrast to the earlier paper, here we do not follow Dorn's nomenclature, and refer to the loose connective tissue surrounded by the serosa and the muscular layer by the name of subserosa, as part of the tunica externa, rather than by the name of submucosa. According to the classification adopted by us, the tunica interna comprises the muscular layer and the mucosa.

Swimbladders intended for histological processing were placed into Bouin's solution in their entirety for some minutes, then were cut through at the affected part or transversely in the middle, and the smaller parts were again fixed in Bouin's solution for 4 hours. The materials were embedded in paraffin wax and cut into 4 μm thick sections. The preparations were stained with haematoxylin-eosin for general information, with picrosirius stain to study collagenic fibres, by Brown-Brenn and by Ziehl-Neelsen for bacteria, by Perls for haemosiderin, and by the periodic acid–Schiff (PAS) reaction for mucous cells. Some sections were treated with 10% H_2O_2 solution for one hour, then stained also by the method of Perls and by the Oil-red procedure.

Electron microscopic examination involved the subsequent processing of material that had been previously fixed in Bouin's solution and embedded in paraffin wax.

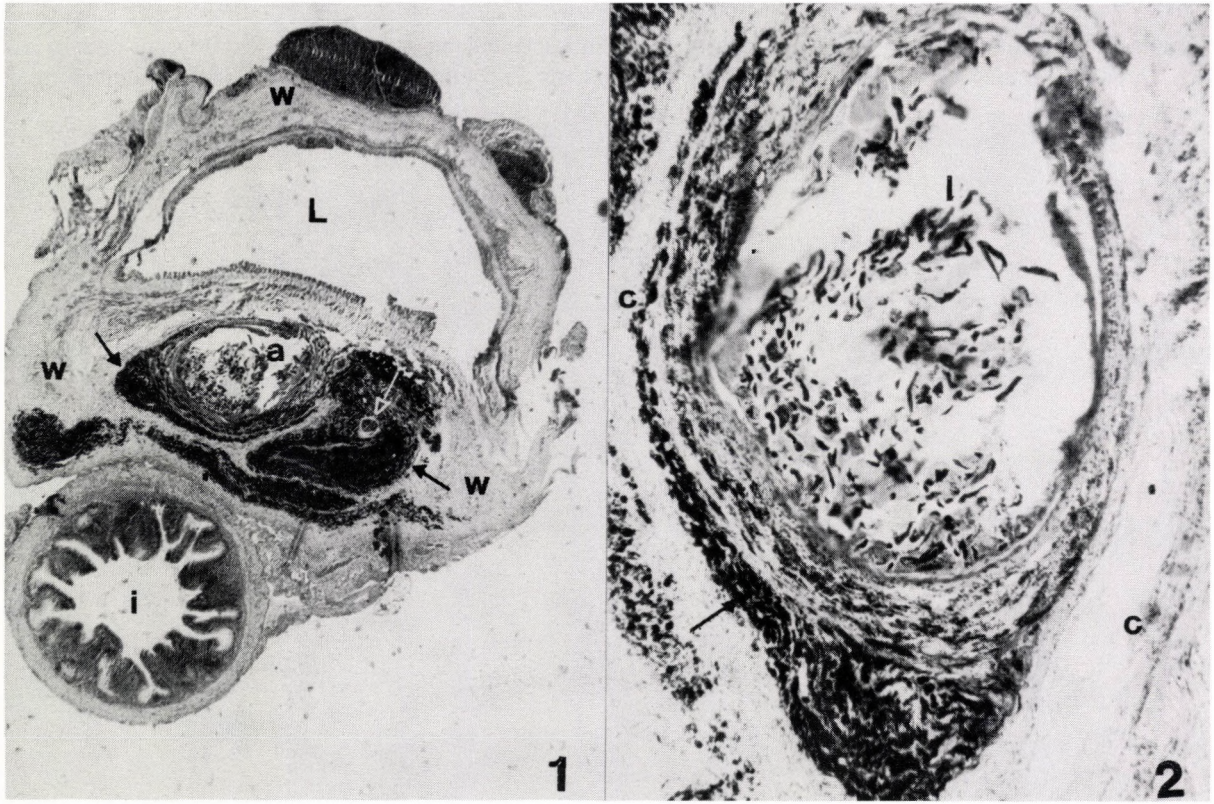


Fig. 1. Cross-section of the swimbladder and a part of the intestine (i). Note an abnormally located female *Anguillicola crassus* (a) in the wall (w) of the swimbladder. The worm was translocated here from the lumen (L) due to the rupture of the muscular layer and mucosa. An adult (a) and a 4th stage larva (empty arrow) are surrounded by melanomacrophages (arrow). Haematoxylin and eosin (H. and E.), × 35

Fig. 2. Enlarged picture of the extraluminal *A. crassus* female shown in Fig. 1. Second stage larvae (l) fill the body of the helminth. The connective tissue of subserosa (c) around the worm is infiltrated by melanomacrophages (arrow). H. and E., × 130

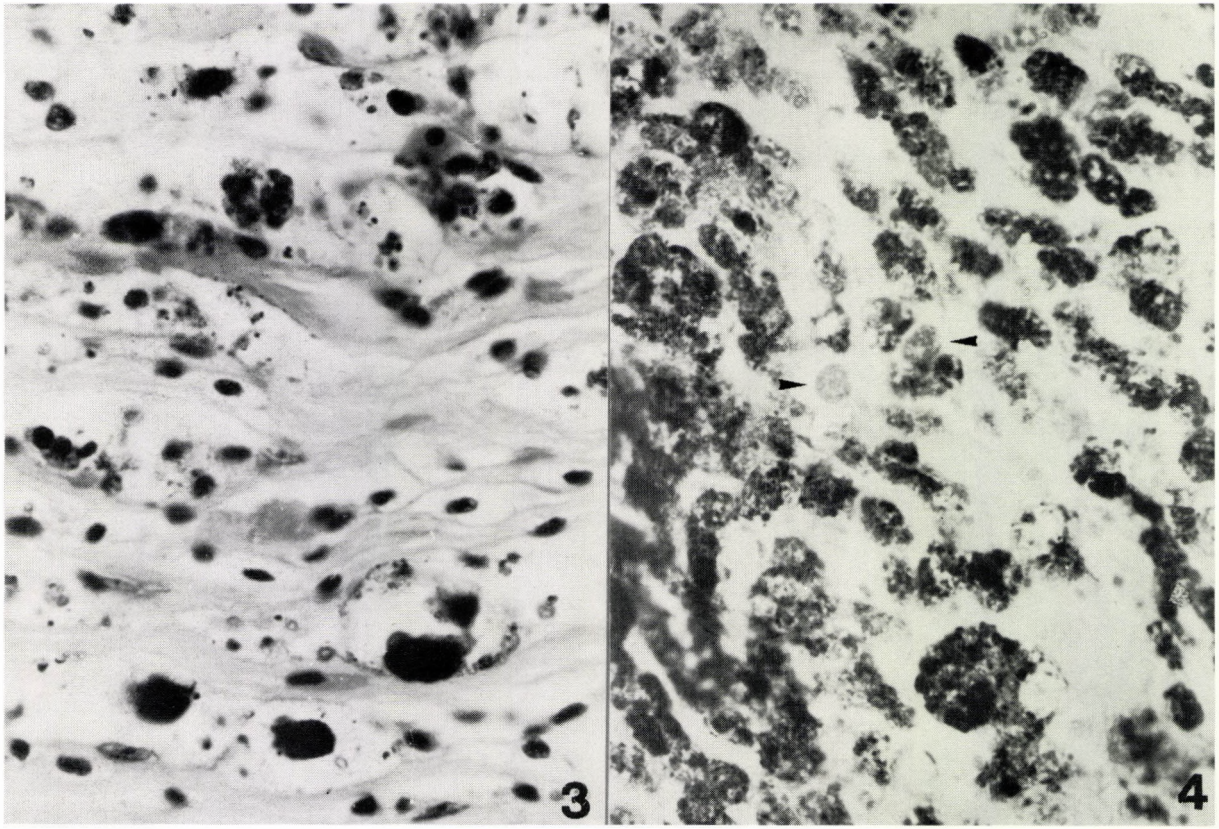


Fig. 3. Melanin-containing macrophages among connective tissue cells filling the lacunas of the subserosa. H. and E., × 1200
Fig. 4. Haemosiderin (arrow-head) in the melanomacrophages. Perls staining after bleaching with H_2O_2. × 800

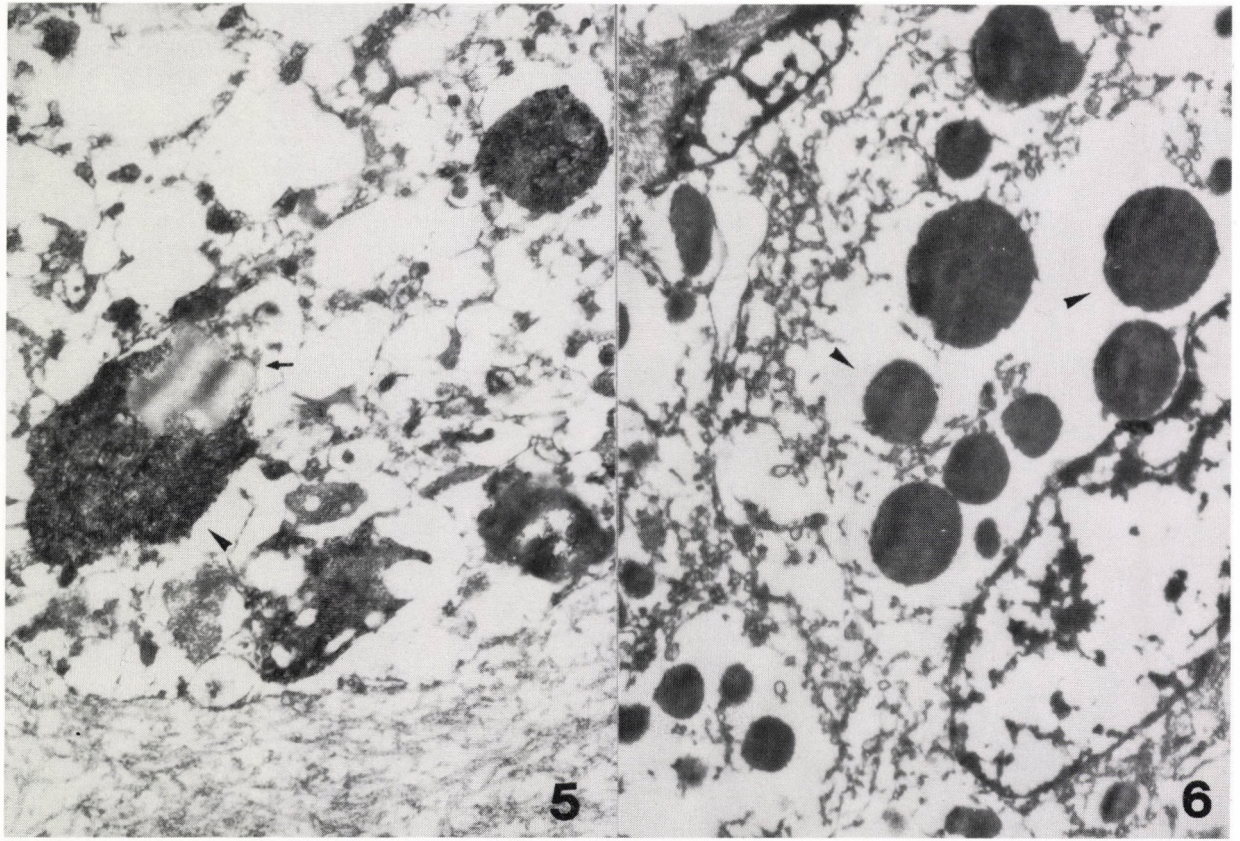


Fig. 5. Electron microscopic section of a macrophage. Inside the cytoplasm note amorphous bodies containing haemosiderin (arrow-head) and lipoid material (arrow). The round body is built up of pigment. × 12,800

Fig. 6. Electron microscopic section of a melanomacrophage. Round-shaped melanin-pigmented material (arrows) is located in the cytoplasm. × 12,800

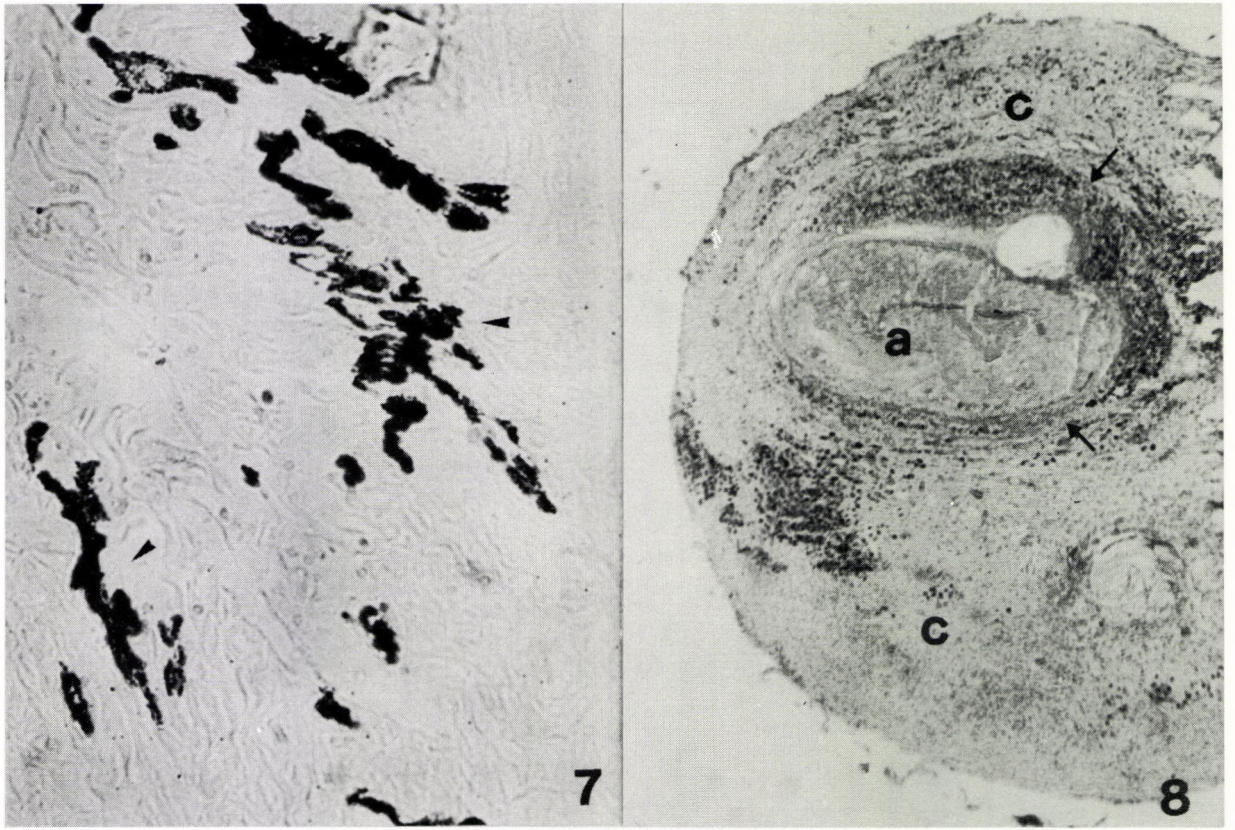


Fig. 7. Melanocytes (arrow-head) in unstained histological section made from the swimbladder serosa. × 800
Fig. 8. Necrotized extraluminal *Anguillicola* adult (a) located in the connective tissue (c) of the posterior part of the swimbladder. The worm is surrounded by melanomacrophages (arrow). H. and E., × 28

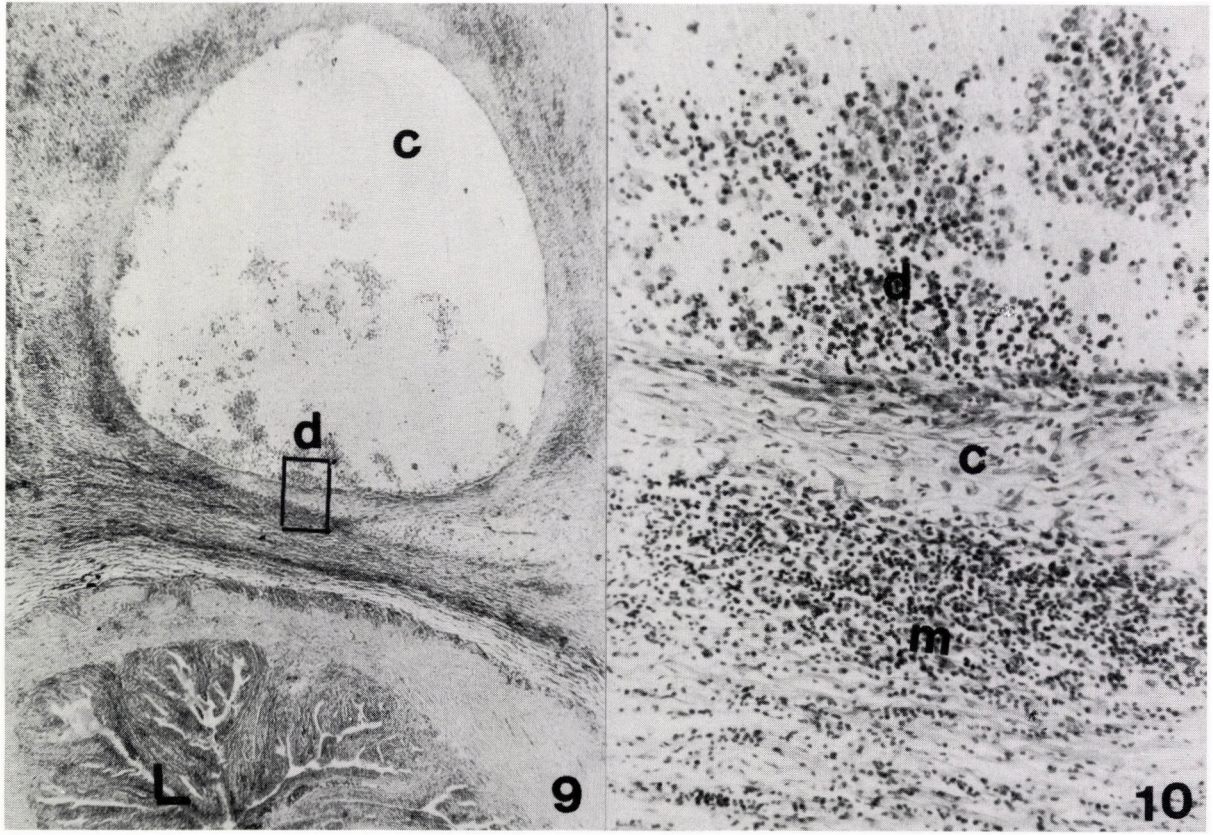


Fig. 9. Remnants of an extraluminal female *A. crassus.* In the cyst (c) the place of the lysed parasite is filled by serum and cell debris (d) of parasite origin. The lumen (L) of the swimbladder is inapparent. H. and E., × 25

Fig. 10. Enlarged part of Fig. 9. No cuticle of the worm is seen. Cell debris (d) of the worm is in direct contact with connective tissue of the subserosa. The latter has been infiltrated by mononuclear cells (m). H. and E., × 250

Results

Molnár et al. (1991) have already reported on the massive and selective eel mortality that occurred in Lake Balaton in the summer of 1991. Eel mortality occurred also in the summer of 1992 although losses were not as high. During both of these outbreaks, highly intensive *Anguillicola crassus* infection was established as the only factor that could account for the observed mortality. Practically all eel specimens derived from the lake were either heavily infected by *Anguillicola* nematodes or showed the signs of previous infection (Molnár et al., 1993). In addition to the damage done by adult helminths located in the swimbladder and by 3rd and 4th stage larvae migrating in the swimbladder wall, further examinations revealed lesions that are not necessarily associated with the condition caused by *A. crassus*, and which presumably resulted from rupture of the swimbladder wall, as mentioned by Liewes and Schaminee-Main (1987) and Kamstra (1990) as the most severe form of anguillicolosis.

Signs indicative of swimbladder lesions (extraluminally located helminths and intensive pigment accumulation) were observed only in the period between July and October. At that time, 27 out of the 344 eels showed such lesions, and all of them also had histological evidence suggestive of a mechanical injury of the swimbladder wall (extraluminally located helminths or 2nd stage larvae). Histological examination confirmed that in cases when the thickened swimbladder wall showed intensive pigment accumulation, helminths abnormally entering the swimbladder wall or disrupted helminth debris could be found in the subserosa, almost exclusively in the caudal part of the swimbladder.

In acute cases of extraluminal helminths, parasites originating from the lumen and containing 2nd stage larvae typically were present in the subserosa of the swimbladder wall (Fig. 1). However, breaks in the tunica interna that could have acted as the site of helminth penetration were not seen in such acute cases. At that time the loose connective tissue containing the helminth was demarcated from the empty lumen by the mucous membrane and muscular layer of the swimbladder. The cuticle of the helminths and the 2nd stage larvae within their body were easily discernible and stained intensely with haematoxylin (Fig. 2). The helminths were surrounded by compact connective tissue consisting of 4–5 cell rows. By picrosirius staining this tissue was shown to contain mature collagenic fibres. These latter were surrounded by a less compact connective tissue layer amongst which were located macrophages containing a brown-black pigment (Fig. 3). In areas of the subserosa more distant from the helminths, a loose connective tissue rich in lacunas was seen (Figs 1, 2 and 3). It was interesting to note that in such cases pigment-containing macrophages accumulated also around the 3rd and 4th stage larvae arrested in their migration (Fig. 1). Less pronounced pigment accumulation was occasionally seen in the tunica propria, as well as in the serosa.

No bacteria were seen in the connective tissue layer surrounding the parasites using Brown-Brenn and Ziehl-Neelsen stains. By Perls staining, no haemosiderin was detectable. Some of the brown-black pigment stained red with Ziehl-Neelsen and dark red with PAS. Hydrogen peroxide treatment bleached the pigment contained by macrophages surrounding the parasite. Subsequently part of the bleached pigment stained red with oil-red, and haemosiderin was detected in some macrophages by Perls stain (Fig. 4).

Electron microscopy revealed granules composed of haemosiderin and a lipoid material in the cytoplasm of some macrophages, and melanin granules were also detected (Figs 5 and 6).

Black pigment was found not only in macrophages but also in melanocytes (Fig. 7). These cells were located in the blood vessel walls, in the tunica propria and in the serosa either individually or in groups. They differed from the macrophages (Fig. 3) both in shape and in the fine-grained black pigment they contained which did not stain by Ziehl-Neelsen nor by PAS, was not affected by hydrogen peroxide treatment, and was negative even by the Perls stain.



Fig. 11. Subserosa of the swimbladder. Giant cells of foreign body type (empty arrows) have been formed around 2nd stage larvae (arrow) penetrating this layer from the lumen through the damaged tunica interna. H. and E., × 250

In more advanced cases the helminth was located in the caudal part of the swimbladder, distant from the lumen (Fig. 8). These helminths had already died; both their cuticle and their inner organs had completely degenerated and their former structure was completely unrecognizable. Areas containing necrotic helminths were surrounded by a compact connective tissue consisting of 4–5 pale-staining layers. On the side of the demarcating connective tissue facing the helminth there was nuclear debris, while on its outer surface masses of mononuclear cells were seen. That layer was surrounded by a broad pigmented zone also in that case, which — as in the case described above — consisted of loose connective tissue containing masses of melanomacrophages that had entered the serous lacunas. That pigment also stained as described earlier.

In the most advanced cases, evidence for the involvement of the abnormally located helminths could be inferred only from the presence of worm debris in the connective tissue capsule or from the cyst that developed in the helminth's place (Fig. 9). In those cases the lumen of the swimbladder was completely collapsed and contained no air. The subserosa contained a cyst that had an enormous lumen and a wall consisting of 6–8 layers of compact connective tissue. Besides the amorphous and pale-staining stroma, the cyst contained probably helminth-derived cells of degenerated cytoplasm and nuclear debris (Fig. 10). Here and there, the outlines of helminth eggs were still discernible in the lumen of the cyst. The innermost layer of the connective tissue capsule was degenerated. Numerous mononuclear elements had appeared in the compact connective tissue, and these cells could be seen in both the subserosa and the serosa. Macrophages appeared also in the interstitial spaces; however, these contained only brown pigment. The pathological process was aggravated by the penetration into the serosa and subserosa of numerous 2nd stage larvae which were surrounded by macrophages, mononuclear cells and also by giant cells (Fig. 11). The pigment showed staining properties similar to those reported for the pigment contained by the macrophages.

Discussion

These cases demonstrate that healing lesions of a recovering severe anguillicolosis are frequently accompanied by complications associated with pronounced damage to the swimbladder wall. Such lesions may develop primarily in the wake of the swimbladder wall rupture described by Liewes and Schaminee-Main (1987) and Kamstra (1990). Thomas and Ollevier (1992) frequently observed thickened (fibrotic) swimbladders but failed to observe swimbladder rupture and helminths freely located in the abdominal cavity. In the cases examined in this study, we did not observe the swimbladder rupture or atrophy, as did Liewes and Schaminee-Main (1987) and Kamstra (1990). However, based upon the adult helminths seen in extraluminal position and the presence of 2nd stage larvae in the tissues, we as-

sume that the local injury of the swimbladder epithelium and muscular layer is much more common than indicated from our dissections. Tissue injury and degeneration of the tunica interna of the swimbladder probably enable helminths, eggs and larvae situated in the lumen to penetrate into the loose, extensible connective tissue, driven by the enhanced intraluminal pressure. Accompanied by the penetrating helminths are tissue and parasite debris and other inflammatory breakdown products which are also translocated into intercellular position. Initially only the tissue debris while later on also the necrotic parasites, 2nd stage larvae and eggs behave as extraneous material, and elicit a foreign-body type giant cell reaction. This stimulates the eel's immune response to segregate and then remove the foreign materials. Tissue enzymes are likely to play the main role in the breakdown of helminths. The first sign of these reactions is the formation of a limiting connective tissue layer around the parasites, with the presence of pigment-containing macrophages that assemble to resorb and remove the foreign material. As the formation of the collagenic fibres takes at least 7–9 days, the pathological processes observed must have started earlier than that. However, parasites could not have penetrated the swimbladder wall more than 8–10 weeks earlier, as during that amount of time connective tissue tends to completely fill up tissue gaps in the destroyed area.

The formation and nature of the pigment deposited in the macrophages are not known in sufficient detail. In agreement with Roberts (1975) we believe that cells containing black pigment in fish (and thus also in the eel) can be classified into at least two types. The black pigment found in the serosa covering the swimbladder, in the blood vessel walls and in the tunica propria is situated in melanocytes, cells which get to these sites as a result of processes associated with aging. In contrast, the brown-black pigment found in the vicinity of the parasites results from the activity of macrophages involved in resorption and removal. That pigment mainly consists of phagocytosed lipoid-like substances, lipofuscin and a small amount of haemosiderin. The degree of pigmentation depends on how advanced the process is. In new haemorrhagic processes always the haemosiderin-containing macrophages while in more chronic cases those containing a darker pigment are encountered more frequently. The latter probably contain more or less oxidized lipofuscin, as suggested by Roberts (1975). As a result of the host reaction induced by the adult helminths and by the 2nd stage larvae, in such cases the 3rd and 4th stage larvae migrating in the swimbladder also undergo necrosis and become surrounded by pigment-containing macrophages. In all likelihood, immunobiological events may be involved in the processes described; this hypothesis, however, requires further study. Although areas infiltrated by mononuclear cells can undoubtedly be found also around the helminths, the appearance of these cellular elements indicates primarily the presence of 2nd stage larvae that have penetrated the tissues. In severe infection, foreign body type giant cells appear in the areas infiltrated by mononuclear cells. These cells indicate injury of the limit-

ing host cells. However, the possible involvement of bacterial activity can neither be ruled out, even if no bacteria could be demonstrated by Braun-Brenn and Ziehl-Neelsen staining.

Acknowledgements

During the work of collecting, dissection and processing the senior author received substantial help from his immediate colleagues, Csaba Székely, Ferenc Baska and Ms Emese Papp, which is gratefully acknowledged. The work was supported by the National Research Fund (OTKA), contract no. 1985.

References

Belpaire, C., De Charleroy, D., Thomas, K., Van Damme, P. and Ollevier, F. (1989): Effect of restocking on the distribution of the swimbladder nematode *Anguillicola crassus* in Flanders, Belgium. J. Appl. Ichthyol. **5,** 151–153.

Boon, J. H., Lokin, C. J. A., Ceusters, R. and Ollevier, F. (1989): Some properties of the blood of European eel (*Anguilla anguilla*) and the possible relationship with *Anguillicola crassus* infestations. Aquaculture **76,** 203–208.

Boon, J. H., Augustijn, H., Cannaerts, V. M. H., Lokin, C. J. A., Machiels, M. A. M. and Ollevier, F. (1990*a*): The suitability of experimental inoculations with infective larvae of *Anguillicola crassus* and their effect on the growth and mortality of the European eel (*Anguilla anguilla*). Aquaculture **87,** 111–120.

Boon, J. H., Cannaerts, V. M. H., Augustijn, H., Machiels, M. A. M., De Charleroy, D. and Ollevier, F. (1990*b*): The effect of different infection levels with infective larvae of *Anguillicola crassus* on haematological parameters of European eel (*Anguilla anguilla*). Aquaculture **87,** 243–253.

Boon, J. H., Zuxu, Y. and Booms, G. H. R. (1990*c*): Effects of *Anguillicola crassus* and *Trypanosoma granulosum* infection on peripheral blood cells of European eel (*Anguilla anguilla* L.). Bull. Eur. Ass. Fish Pathol. **10,** 143–145

De Charleroy, D., Grisez, L., Thomas, K., Belpaire, C. and Ollevier, F. (1990): The life cycle of *Anguillicola crassus*. Dis. aquat. Org. **8,** 77–84.

Dupont, F. and Petter, A. J. (1988): Anguillicola, une épizootie plurispécifique en Europe. Apparition d' *Anguillicola crassa* (Nematoda, Anguillicolidae) chez l'Anguille europeene (*Anguilla anguilla*) en Camargue, Sud de la France. Bull. Fr. Péche Piscic. **308,** 38–41.

Hartmann, S. (1987): Schwimblassenwürmer beim Aal. Fischer & Teichwirt **38,** 2–3.

Haenen, O. L. M. and van Banning, P. (1991): Experimental transmission of *Anguillicola crassus* (Nematoda, Dracunculoidea) larvae from infected prey fish to the eel, *Anguilla anguilla*. Aquaculture **92,** 115–119.

Haenen, O. L. M., Grisez, L., De Charleroy, D., Belpaire, C. and Ollevier, F. (1989): Experimentally induced infections of European eel *Anguilla anguilla* with *Anguillicola crassus* (Nematoda, Dracunculoidea) and subsequent migration of larvae. Dis. aquat. Org. **7,** 97–101.

Höglund, J., Andersson, J. and Hardig, J. (1992): Haematological responses in the European eel, *Anguilla anguilla* L., to sublethal infestation by *Anguillicola crassus* in a thermal effluent of the Swedish Baltic. J. Fish Dis. **15,** 507–514.

Höglund, J. and Thomas, K. (1992): The black goby, *Gobius niger*, as a potential paratenic host for the parasitic nematode *Anguillicola crassus* in a thermal effluent of the Baltic. Dis. aquat. Org. **13,** 175–180.

Kamstra, A. (1990): Anguillicola in Dutch eelfarms; current state. EIFAC (FAO) Working Party on Eel, Oporto, Portugal, 29 May to 3 June 1989, 7 pp.

Kennedy, C. R. and Fitch, D. J. (1990): Colonization, larval survival and epidemiology of the nematode *Anguillicola crassus,* parasitic in the eel, *Anguilla anguilla,* in Britain. J. Fish Biol. **36,** 117–131.

Koie, M. (1988): Parasites in eels, *Anguilla anguilla* (L.), from eutrophic Lake Estrum (Denmark). Acta Parasit. Polon. **33,** 89–100.

Koops, H. and Hartmann, F. (1989): *Anguillicola* infestations in Germany and in German eel imports. J. Appl. Ichthyol. **5,** 41–45.

Liewes, E. W. and Schaminee-Main, S. (1987): Onderzoek naar de effecten van de parasiet *Anguillicola crassa* op de ontwikkeling van de paling (*Anguilla anguilla*) in een zout water palingmesterij. Internal Report, Texvis B.V., Den Burg Texel, The Netherlands.

Mellergaard, S. (1988): Ålens svommeblæreorm Anguillicola — en ny parasit i den europæiske ålebestand. Nordisk Aquakultur **4,** 50–54.

Molnár, K., Baska, F., Csaba, Gy., Glávits, R. and Székely, Cs. (1993): Pathological and histopathological studies of the swimbladder of eels *Anguilla anguilla* infected by *Anguillicola crassus* (Nematoda: Dracunculoidea). Dis. aquat. Org. **15,** 41–50.

Molnár, K., Székely, Cs. and Baska, F. (1991): Mass mortality of eel in Lake Balaton due to *Anguillicola crassus* infection. Bull. Eur. Ass. Fish Pathol. **11,** 211–212.

Moravec, F. (1992): Spreading of the nematode *Anguillicola crassus* (Dracunculoidea) among eel populations in Europe. Folia parasitol. **39,** 247–248.

Möller, H., Holst, S., Lüchtenberg, H. and Petersen, F. (1991): Infection of eel *Anguilla anguilla* from the River Elbe estuary with two nematodes, *Anguillicola crassus* and *Pseudoterranova decipiens*. Dis. aquat. Org. **11,** 193–199.

Neumann, W. (1985): Schwimmblasenparasit Anguillicola bei Aalen. Fischer & Teichwirt **36,** 322.

Peters, G. and Hartmann, F. (1986): Anguillicola, a parasitic nematode of the swim bladder spreading among eel populations in Europe. Dis. aquat. Org. **1,** 223–230.

Roberts, R. (1975): Melanin containing cells of teleost fish and their relation to disease. In: Ribelin, W. E. and Migaki, G. (eds.) The Pathology of Fishes. University of Wisconsin Press, Madison, Wisconsin, pp. 399–428.

Sprengel, G. and Lüchtenberg, H. (1991): Infection by endoparasites reduces maximum swimming speed of Europaen smelt *Osmerus eperlanus* and European eel, *Anguilla anguilla*. Dis. aquat. Org. **11,** 31–35.

Székely, Cs., Láng, M. and Csaba, Gy. (1991): First occurrence of *Anguillicola crassus* in Hungary. Bull. Eur. Ass. Fish Pathol. **11,** 162–163.

Taraschewski, H., Moravec, F., Lamah, T. and Anders, K. (1987): Distribution and morphology of two helminths recently introduced into European eel populations: *Anguillicola crassus* (Nematoda, Dracunculoidea) and *Paratenuisentis ambiguus* (Acanthocephala, Tenuisentidae). Dis. aquat. Org. **3,** 167–176.

Thomas, K. and Ollevier, F. (1992): Population biology of *Anguillicola crassus* in the final host *Anguilla anguilla*. Dis. aquat. Org. **14,** 163–170.

Van Banning, P and Haenen, O. L. M. (1990): Effects of the swimbladder nematode *Anguillicola crassus* in wild and farmed eel, *Anguilla anguilla*. In: Perkins, F. O. and Cheng, T. C. (eds) Pathology in Marine Science. Academic Press, New York, pp. 317–330.

Van Willigen, J. and Dekker, W. (1989): 1988 update on *Anguillicola* in Dutch outdoor waters. EIFAC (FAO) Working Party on Eel. Oporto, Portugal, 29 May to 3 June 1989, 8 pp.

Acta Veterinaria Hungarica 43 (1), pp. 139–144 (1995)

ULTRASTRUCTURAL OBSERVATIONS ON THE THIRD-GENERATION MEROZOITES OF *Eimeria tenella* IN CHICKS[+]

S. J. BALL[1], P. DASZAK[1*], R. M. PITTILO[2] and C. C. NORTON[3]

[1]Department of Life Sciences, University of East London, Romford Road, London, E15 4LZ, U. K.; [2]School of Life Sciences, Kingston University; [3]Central Veterinary Laboratory, MAFF, Weybridge, Surrey, U. K.

(Received June 2, 1994)

The fine structure of the third-generation merozoites of *Eimeria tenella* is described from caecal enterocytes of chicks 120 h after experimental infections. The merozoites conformed to the general eimerian pattern except that they were bounded by two unit membranes. Young gamonts also conformed to the general eimerian pattern, and were limited by an outer unit membrane and an interrupted inner membrane. Enterocytes invaded by third-generation merozoites showed a loss of microvilli and large blebs that protruded into the caecal lumen. These blebs appeared to contain only cytoplasm and not any cell organelles.

Key words: *Eimeria tenella*, third-generation merozoites, ultrastructure

Tyzzer (1929) described the life cycle of *Eimeria tenella* in chickens and concluded that the majority of second-generation merozoites developed into gamonts and only a minority produced third-generation meronts; these were present at the same time as the sexual stages. Since then, this has been the premise for the interpretation of many experimental studies, including those dealing with ultrastructure. However, some conclusions may need reassessment now that McDonald and Rose (1987) have shown the third-generation merogony to be an obligatory stage in the life cycle of *E. tenella*, and that most, if not all, gamonts are derived from the merozoites of this generation.

The present study was conducted to examine the ultrastructure of the third-generation merozoites, their effect on enterocytes and the appearance of early gamonts derived from these merozoites.

[+] This paper is dedicated to commemorating the 20th anniversary of the death of László Pellérdy, the renowned Hungarian protozoologist

[*] Present address: School of Life Sciences, Kingston University, Penrhyn Road, Kingston upon Thames, Surrey, KT1 2EE, U. K.

0236–6290/95/$ 4.00 © 1995 *Akadémiai Kiadó, Budapest*

Materials and methods

Two Ross Brown light hybrid cockerels were reared coccidia-free from 1 day old and maintained throughout on a ration deficient in vitamin K as described by Ryley and Betts (1973). At 39 days of age the chicks were each orally inoculated with 5,000 sporulated oocysts of the Weybridge strain of *E. tenella.* The birds were killed 120 h after infection by an intracardiac injection of sodium pentobarbitone. Tissues from each caecum were prepared for transmission electron microscopy by standard procedures previously described (Pittilo and Ball, 1979) and sections examined in an AEI Corinth 275 microscope.

Results

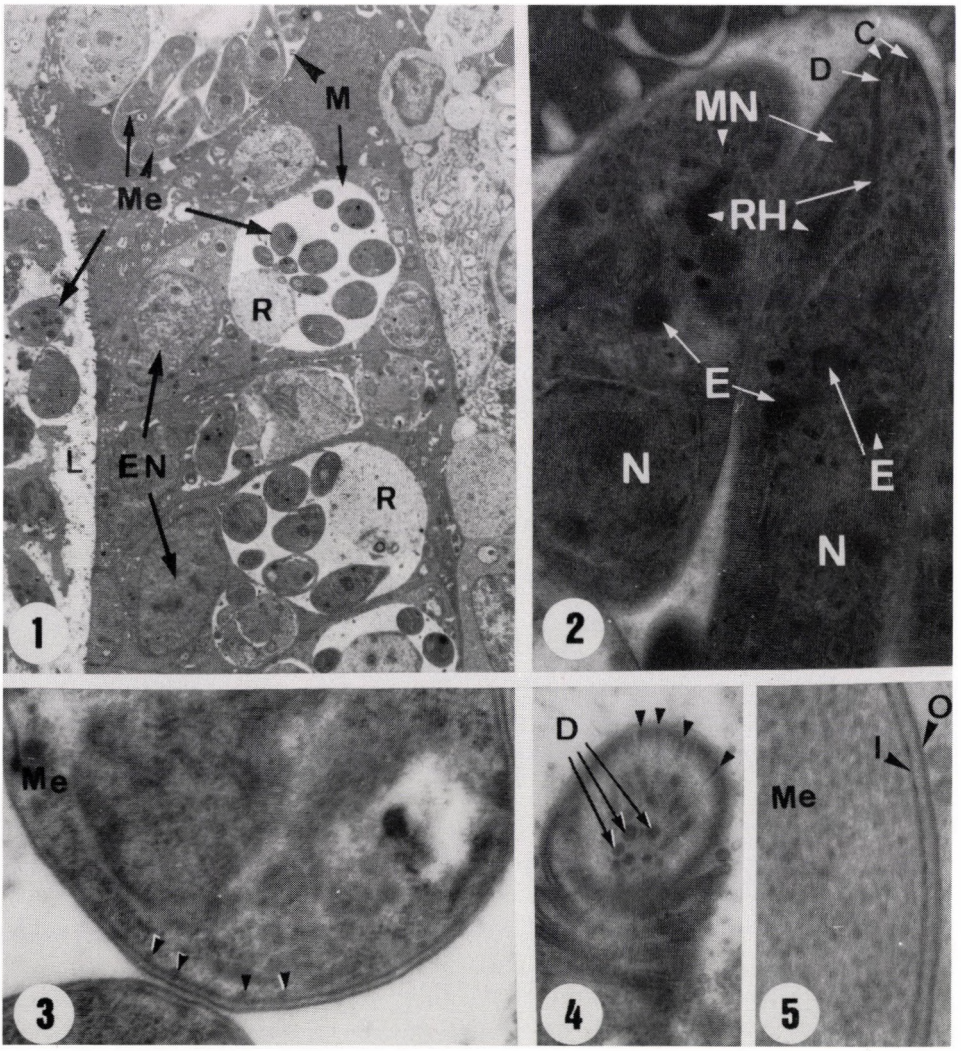
It was not possible to accurately estimate the numbers of various stages present in the segments of caeca examined, but it is our subjective opinion that the third-generation meronts made up the vast majority of stages with relatively few second-generation meronts and gamonts. The third-generation meronts, with a maximum number of 12 merozoites, formed beneath the nucleus of the infected enterocytes (Fig. 1). The merozoites possessed typical eimerian organelles in the anterior regions, such as conoid, micronemes, rhoptries (possibly 6 — Fig. 4), and electron dense bodies (Fig. 2). The merozoites were bounded by two unit membranes (Figs 3 and 5) under which subpellicular microtubules were occasionally evident, but not clearly resolved, in the anterior region (Figs 3 and 4).

Structural changes in enterocytes, after invasion by third-generation merozoites, involved loss of microvilli and protrusions of the cell into the caecal lumen. These bleb-like protrusions appeared devoid of organelles and to only contain cytoplasm (Figs 6 and 7).

Intracellular merozoites were found within parasitophorous vacuoles (Figs 6 and 7). Single third-generation merozoites within enterocytes often had partially emptied rhoptries (Fig. 8).

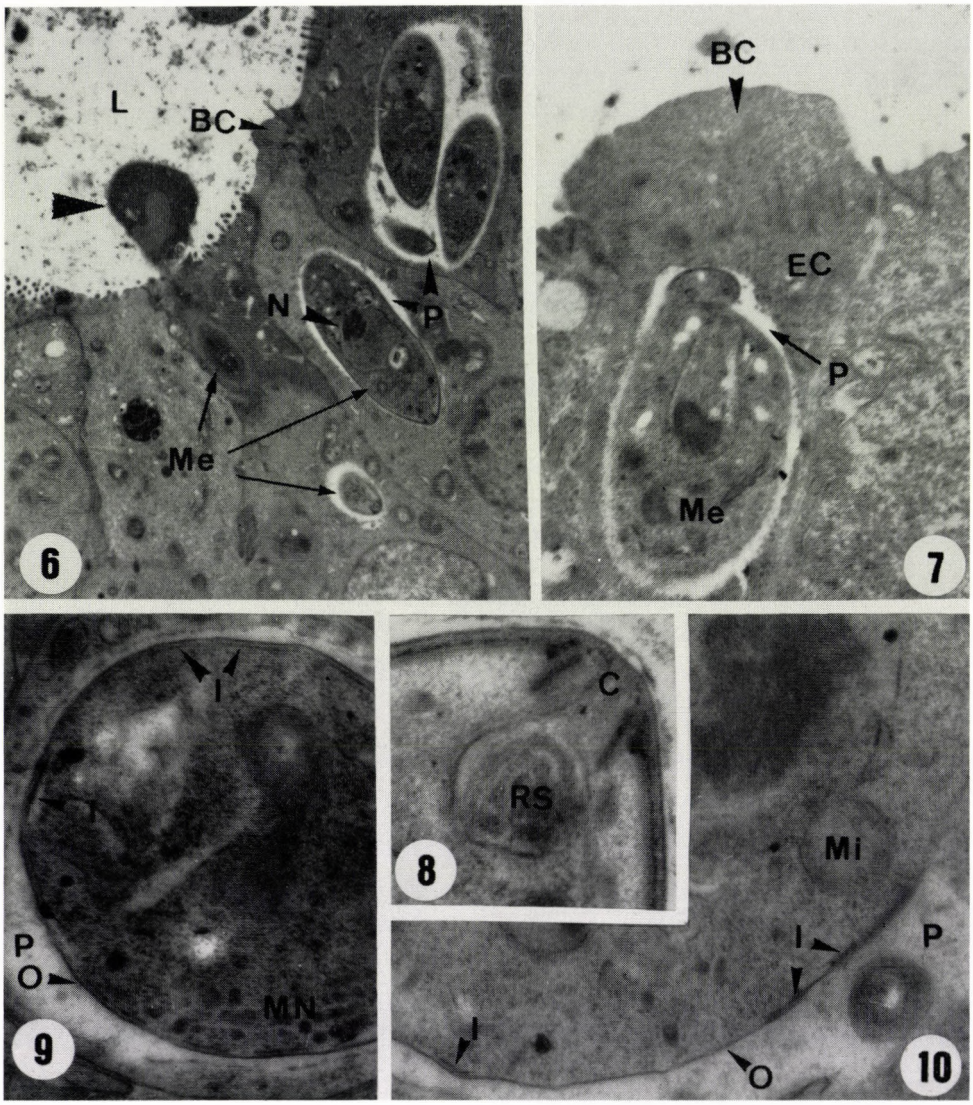
Young gamonts were surrounded by an outer unit membrane and an interrupted inner membrane (Figs 9 and 10).

Abbreviations used in Figures: BC = bulge of cytoplasm; C = conoid; D = ductule of rhoptry; E = electron dense body; EC = enterocyte; EN = enterocyte nucleus; I = inner membrane; L = lumen of caecum; M = meront; Me = merozoite (3rd generation); Mi = mitochondrion of parasite; MN = microneme; N = nucleus of merozoite; O = outer membrane; P = parasitophorous vacuole; R = residual cytoplasm; RH = rhoptry; RS = rhoptry saccule



Figs 1–5. Transmission electron micrographs (TEM) of third-generation meronts and merozoites of *Eimeria tenella*.

Fig. 1. Mature meronts with merozoites cut transversely, obliquely and longitudinally. × 1,600. *Fig. 2.* Longitudinal section of two merozoites showing organelles of the anterior region. × 13,000. *Fig. 3.* Transverse section of a merozoite bounded by two membranes beneath which subpellicular microtubules (4 shown by arrow heads) are just visible. × 5,300. *Fig. 4.* Oblique section through folded anterior end of a merozoite showing microtubules (4 indicated by arrow heads) and a possible six rhoptry ductules (3 labelled) × 11,000. *Fig. 5.* Transverse section of portion of a merozoite bounded by two unit membranes. × 16,000



Figs 6–10. TEM of mature third-generation merozoites and young gamonts of *Eimeria tenella*. *Fig. 6.* Enterocytes invaded by merozoites and showing cytoplasmic bulge (BC) and apparent extruded cytoplasm (unlabelled arrow head). × 2,500. *Fig. 7.* Abnormal bulge of cytoplasm, containing no apparent organelles, projects into lumen from invaded enterocyte. No microvilli are visible on the surface of the protrusion. × 6,200. *Fig. 8.* Anterior portion of an intracellular merozoite showing conoid and partially emptied rhoptry saccule. × 24,400. *Fig. 9.* Young gamont showing interrupted inner membrane. × 23,800. *Fig. 10.* Presumed early macrogamete showing sparse remains of the original merozoite inner membrane. × 11,000

Discussion

In many species in the Eimeriidae, a specific number of asexual generations occur before gametogony. In normal development of *E. tenella* it had previously been considered that two generations of merogony preceded the sexual stages. The fine structure of the merozoites and gamonts of eimerians has been well documented (see Scholtyseck, 1973; Chobotar and Scholtyseck, 1982; Ball and Pittilo, 1990). No detailed ultrastructural information has been reported for the third-generation meronts and merozoites of *E. tenella* although they have been referred to occasionally (McLaren and Paget, 1968; McLaren, 1969; Hoppe, 1976).

Third-generation merozoites develop by a process of ectomerogony, called budding by Tyzzer (1929), which results in a relatively large amount of unused or residual cytoplasm. The structure of the merozoites is generally typical of eimerians except that subpellicular microtubules were usually difficult to resolve and the merozoite was bounded by two unit membranes rather than an outer single membrane and an inner double membrane. In transformation of the merozoites into gamonts the cell boundary of the earliest sexual stages is derived from the pellicle of the merozoite. Young gamonts have been reported to be limited by one or two unit membranes only (McLaren, 1969; Doens-Juteau and Senaud, 1974) or, usually in the case of macrogametes, by an outer single membrane underlain by remnants of the inner membrane complex of the merozoite (Hoppe, 1976). Our findings also show that the young macrogamonts are bounded by a single membrane, with an underlying interrupted membrane, apparently representing the inner membrane of the third merozoite and not remnants of an inner membrane complex, that is a double membrane, as would be expected if the sexual stages derived from second-generation merozoites.

The appearance of enterocytes invaded by third merozoites was similar to that recorded after penetration by first-generation merozoites of *E. tenella* (Daszak et al., 1993). After invasion by either stage the apical cytoplasm of the invaded cell protruded into the lumen and formed an homogeneous bulge devoid of organelles and microvilli. Whether this is a general reaction of enterocytes to the invasion by other coccidial merozoites is not known, but it differs entirely from the penetration of sporozoites and merozoites *in vitro*, where invagination of the membrane of the cultured cell occurs unaccompanied by the cytoplasmic protrusions described here.

Acknowledgement

We are indebted to F. Hoffmann-La Roche Limited (Basle, Switzerland) for financial support of P. Daszak and to Dr G. M. Weber of Hoffmann-La Roche for his interest in our work.

References

Ball, S. J. and Pittilo, R. M. (1990): Structure and Ultrastructure. In: Long, P. L. (ed.) Coccidiosis of Man and Domestic Animals. CRC Press, Boca Raton, pp. 17–41.

Chobotar, B. and Scholtyseck, E. (1982): Ultrastructure. In: Long, P. L. (ed.) The Biology of the Coccidia. University Park Press, Baltimore, pp. 101–165.

Daszak, P., Ball, S. J., Pittilo, R. M. and Norton, C. C. (1993): Ultrastructural observations on caecal epithelial cells invaded by first-generation merozoites of *Eimeria tenella in vivo*. Ann. Trop. Med. Parasit. **87,** 359–364.

Doens-Juteau, O. and Senaud, J. (1974): Ètude ultrastructurale de la paroi du macrogamète et de l'oocyste chez *Eimeria tenella* (Railliet et Lucet, 1891) coccidie Eimeriidae, parasite de l'intestin des poulets. Protistologica **10,** 261–270.

Hoppe, G. (1976): The ultrastructure of early generation development and later schizonts of *Eimeria tenella* in the chicken. Protistologica **12,** 169–181.

McDonald, V. and Rose, M. E. (1987): *Eimeria tenella* and *Eimeria necatrix*: a third generation of schizogony is an obligatory part of the developmental cycle. J. Parasit. **73,** 617–622.

McLaren, D. J. (1969): Observations on the fine structural changes associated with schizogony and gametogony in *Eimeria tenella*. Parasitology **59,** 563–574.

McLaren, D. J. and Paget, G. E. (1968): A fine structural study of the merozoite of *Eimeria tenella* with special reference to the conoid apparatus. Parasitology **58,** 561–571.

Pittilo, R. M. and Ball, S. J. (1979): The fine structure of the developing macrogamete of *Eimeria maxima*. Parasitology **79,** 259–265.

Ryley, J. F. and Betts, M. J. (1973): Chemotherapy of chicken coccidiosis. Adv. Pharmacol. Chemother. **11,** 221–293.

Scholtyseck, E. (1973): Ultrastructure. In: Hammond, D. M. and Long, P. L. (eds) The Coccidia. University Park Press, Baltimore, pp. 81–144.

Tyzzer, E. E. (1929): Coccidiosis in gallinaceous birds. Amer. J. Hyg. **10,** 269–383.

Acta Veterinaria Hungarica 43 (1), pp. 145–151 (1995)

EOSINOPHILS IN LYMPH NODES OF COWS INFECTED BY BOVINE LEUKAEMIA VIRUS

M. LEVKUT[1], Maria LEVKUTOVÁ[2], V. KONRÁD[1] and M. POLÁČEK[3]

[1]Department of Pathological Anatomy, University of Veterinary Medicine, 04181 Košice, Slovakia; [2]Department of Infectious and Tropical Diseases, University of Veterinary Medicine, 04181 Košice, Slovakia; [3]Department of Computer Technique, Biomathematics and Economics, University of Veterinary Medicine, 04181 Košice, Slovakia

(Received May 25, 1994)

Prescapular lymph nodes from 109 animals positive to bovine leukaemia virus (BLV) were evaluated in this study. Lymph nodes of 81 animals (74.3%) showed an increased number of eosinophils (Eo) in a variety of pathological reactions. Eo counts in T-zonal hyperplasia (47 cases, 43.1%) and mixed hyperplasia (13 cases, 11.9%) were significantly higher ($P < 0.05$ and $P < 0.01$, respectively) than in the control group. Similarly, a significantly increased number of blood eosinophils (BEo) was observed in cows with diffuse infiltration by Eo in the lymph nodes as compared to the number of BEo in cows serologically positive to BLV but without morphological changes in the lymph nodes and in cows which were serologically negative ($P < 0.05$ and $P < 0.01$, respectively). The possible role of eosinophilic granulocytes in the regulation of immune response to enzootic bovine leukaemia (EBL) is discussed.

Key words: Cattle, enzootic bovine leukaemia, lymph nodes, eosinophils

Eosinophils are potent proinflammatory cells that play a crucial role in the defense mechanisms of the body, particularly against helminths in parasitic infections. They are also associated with many hypersensitivity diseases, and are very likely to be involved in host tissue damage (Gennaro et al., 1991). Hypereosinophilic syndrome is frequently associated with human malignant lymphoma which is induced by the T lymphotrophic virus (HTLV-I) (Prin et al., 1988; Vukelja et al., 1988). Eosinophilia appears to be a good prognostic indicator of Hodgkin's lymphoma (Vaughan Hudson et al., 1987) and correlates with an extensive deposition of eosinophilic peroxidase in the affected tissues (Samozuk et al., 1986). It is widely known that peripheral blood eosinophilia and eosinophilic infiltration, predominantly of the lymph nodes, occur before the clinical manifestation of enzootic bovine leukaemia (EBL). A slight to marked eosinophilic infiltration was observed in the medulla, hilus, capsule and trabeculae of the examined lymph nodes (Ohshima et al., 1982). EBL is a chronic disease of the haemopoietic system affecting the lymphatic tissue. Bovine leukaemia virus (BLV) is the causa-

0236–6290/95/$ 4.00 © 1995 *Akadémiai Kiadó, Budapest*

tive agent of this disease. This retrovirus is structurally related to human T lymphotrophic viruses (HTLV-I and HTLV-II) (Burny et al., 1988).

Our previous study divided pathomorphological reactions of lymph nodes of BLV-infected animals into individual groups (Levkut et al., 1993) on the basis of the criteria used for the examination of human lymph node biopsies. The purpose of this study was to examine and quantify eosinophilic infiltration in the lymph nodes as well as in blood, in relationship to individual pathomorphological reactions in the lymph nodes of BLV-infected cattle.

Materials and methods

Animals. Prescapular lymph nodes from 109 cows serologically positive to BLV and from 10 serologically negative control cows were used in the study. The majority of the animals belonged to the Black Spotted and Slovak Spotted breeds (4–6 years of age).

Detection of BLV antibodies. An immunodiffusion test (Bioveta Nitra, Slovakia) was used to detect BLV antibodies.

Staining of blood smears. Blood smears were stained panoptically by Pappenheim.

Processing of histological material. The removed material was processed using a standard procedure, i.e. fixed in 10% neutral formalin, and embedded in paraffin. Sections of a thickness of 5–6 μm were stained with haematoxylin-eosin and by Giemsa.

Evaluation criteria. Eosinophils were counted in the paracortical area and the medullar sinuses, and in the case of pulp proliferation among plasma cells. The degree of infiltration by eosinophils (Eo) in the lymph nodes was graded by one (+) to three (+++) pluses.

\+ focal Eo infiltration with the presence of 50–224 eosinophilic granulocytes in 50 light microscopic visual fields at a magnification of × 1000.

++ focal Eo infiltration with 225 or more eosinophilic granulocytes in 50 light microscopic visual fields at a magnification of × 1000.

+++ diffuse Eo infiltration with 225 or more eosinophilic granulocytes in 50 light microscopic visual fields at a magnification of × 1000.

Statistical analysis. The statistical significanses of the data were evaluated by Student's *t*-test.

Results

Macroscopic examinations of 109 slaughtered animals did not reveal any neoplastic processes of predilective organs or parasitic invasion in these animals.

Some examined prescapular lymph nodes showed moderate enlargement. Visible follicular hyperplasia on the cut surface, or a vague structure was observed in the enlarged nodes. Histological examination, which divided the pathomorphological reaction of the lymph nodes into five groups, has been reported earlier (Table 1, Levkut et al., 1994).

Table 1

Pathomorphological reactions of prescapular lymph nodes in 109 cows infected with BLV

Type of reaction	Frequency	
	No. of cases	%
1. B-zonal hyperplasia	23	21.1
2. T-zonal hyperplasia	52	47.7
3. Pulp proliferation	6	5.5
4. Mixed hyperplasia	28	16.5
5. Atrophy	10	9.2

A histological evaluation of eosinophils in the prescapular lymph nodes of 109 animals recorded an increased number of eosinophilic granulocytes in 81 cases (74.31%). The degree of infiltration by Eo for individual morphological reactions in lymph nodes is presented in Fig. 1. A significantly increased density of Eo was observed in T zonal hyperplasia ($P < 0.05$) and in mixed hyperplasia ($P < 0.01$), as compared with the density of Eo in the lymph nodes of the control group (Fig. 1). Eosinophilic infiltration was recognized in these pathomorphological lesions, predominantly in the medullary sinuses, in their surroundings and in the paracortical area. Eosinophils were also frequently observed in the medullary cords between small lymphocytes and plasma cells. In the case of pulp proliferation, these cells were diffused among proliferating plasma cells. Besides the above-mentioned areas, marked tissue eosinophilia (+++) of the hilus was also seen. The correlation between the occurrence of blood eosinophils (BEo) and the serological status of animals for BLV is presented in Fig. 2. A significantly increased number of BEo was observed in cases with diffuse, marked eosinophilic infiltration, compared to the occurrence of BEo in the seropositive animals without morphologic alterations in their lymph nodes ($P < 0.05$) and in BLV seronegative cows ($P < 0.01$).

The correlation between the density of eosinophils in individual morphological changes in the lymph nodes and the blood eosinophilia was not determined.

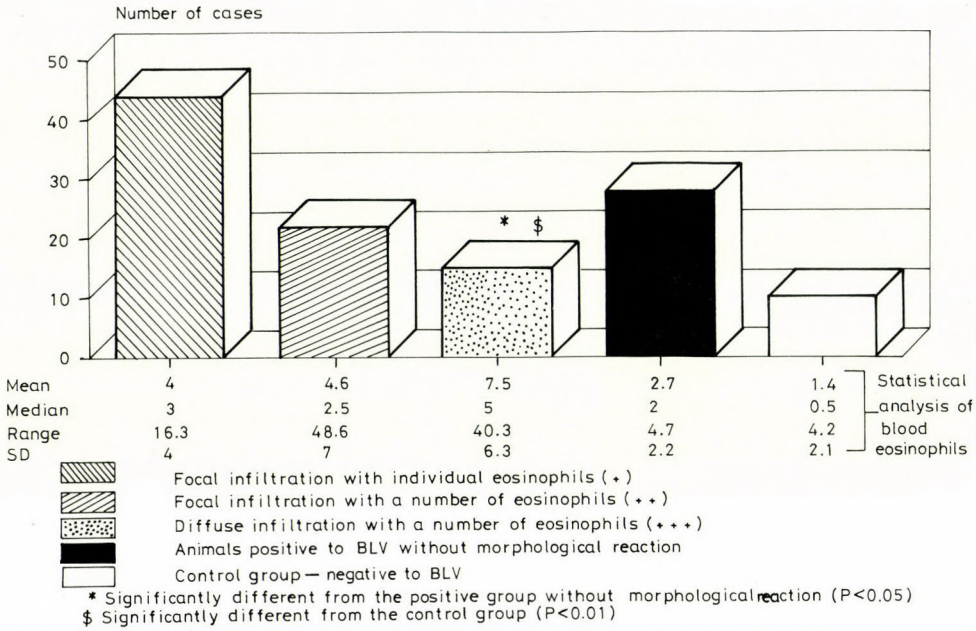


Fig. 1. Number of cases and the density of eosinophils at individual morphological changes in lymph nodes

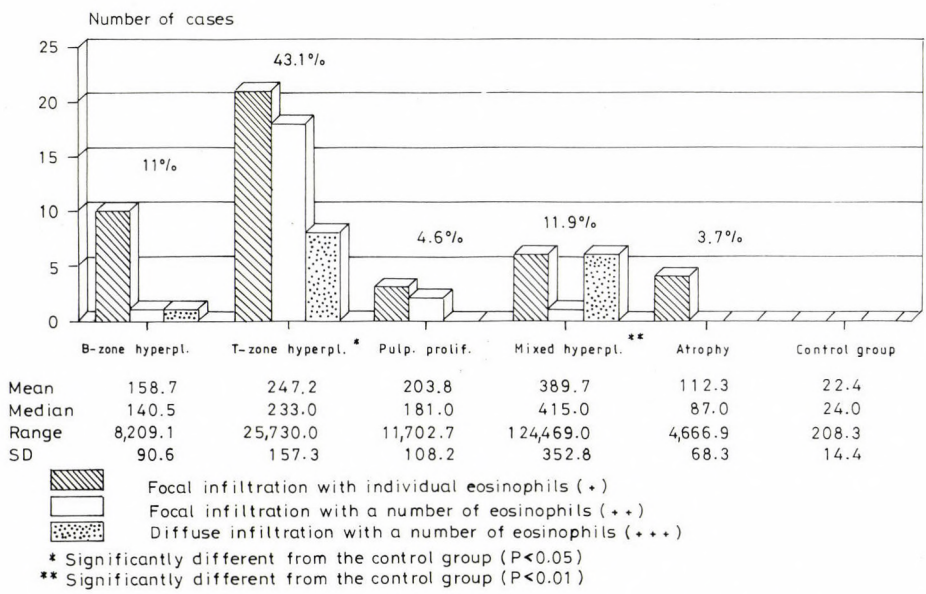


Fig. 2. Relation of blood eosinophils to lymph node eosinophilia

Discussion

In this study, tissue eosinophilia was found in 74.3% of the 109 examined prescapular lymph nodes of BLV-infected cattle. A significant increase of infiltration with eosinophils was observed only in T-zonal hyperplasia and mixed hyperplasia. Numerous investigations in humans have led to the conclusion that eosinophilic infiltration is a T-lymphocytic phenomenon (Aisenberg and Wilkes, 1982; Borowitz et al., 1982). It is assumed that interleukin-2 (IL-2), the product of activated T lymphocytes, enhances the cytolytic abilities of cytotoxic T-cells (Breazile, 1988). Observations moreover indicate that IL-2 induces a cellular secretion of biologically active IL-5, which results in peripheral eosinophilia and extravascular degranulation of eosinophils (Pisani et al., 1991). These authors demonstrated that eosinophil degranulation was confirmed by both increased plasma levels of the eosinophil major basic protein and the presence of that protein in the biopsy material of patients with cancer.

Fujisawa et al. (1990) demonstrated that the nonspecific release of eosinophil granule proteins enchances secretion of IgA and IgG. Numerous EBL studies suggest the qualitative and quantitative changes in immunoglobulin expression (Matthaeus, 1978; Fossum et al., 1988; Levkut et al., 1991).

Eosinophils were present not only in the sinuses but also in the paracortical area and the medullary cords of individual pathomorphological reactions in BLV-infected lymph nodes. A mixture of small lymphocytes and plasma cells was observed in these areas. Eosinophils were only seen among plasma cells in the case of pulp proliferation. The above-mentioned locations of eosinophilic infiltration, noted in our study, indicate a certain relationship between T lymphocytes, eosinophils and plasma cells. This assumption is confirmed by the fact that no significant increase in eosinophilic infiltration was observed in B-zonal hyperplasia and lymph node atrophy, which showed a lower T-zonal reactivity. Results obtained by comparing the extent of eosinophilic infiltration and the level of BEo suggest that a marked infiltration by Eo in lymph nodes is associated with an increased number of BEo in BLV-positive animals. This observation also suggests that we may expect the eosinophilic infiltration of BLV-infected lymph nodes to occur first, followed by a period characterized by an increased level of eosinophils in the blood. This supposition is supported by the fact that cows serologically positive to BLV, without morphological reaction, showed levels of BEo comparable to those seen in the control animals. The mechanism of the increased movement of eosinophils from blood into tissues is unknown at present. However, some chemotactic factors, such as eosinophil chemotactic factors derived from mast cells in anaphylaxis and lamellar activating factors produced by eosinophils, are known. Activated T-lymphocytes also secrete a stimulative eosinophil promotor (Breazile, 1988).

This survey indicates that eosinophilic infiltration occurs in individual pathomorphological reactions of BLV-infected lymph nodes with a significant number of lesions which contain a majority of T-lymphocytes. The study also suggests the primary infiltration of lymph nodes and the possibility of the subsequent development of peripheral eosinophilia.

Additional experimental studies examining the possible relationship between lymphocytes, plasma cells and eosinophils and the regulation of the immune response of an organism to BLV should contribute to the knowledge about the role of eosinophils in this disease.

Acknowledgement

We wish to thank E. Maceková, R. Lengová and Z. Balajtyová for technical assistance.

References

Aisenberg, A. C. and Wilkes, B. M. (1982): Lymph node T cells in Hodgkin's disease. Analysis of suspensions with monoclonal antibody and rosetting techniques. Blood **59,** 522–527.

Borowitz, M. I., Croker, B. P. and Metzger, R. S. (1982): Immunohistochemical analysis of the distribution of lymphocyte subpopulations in Hodgkin's disease. Cancer Treat. Rep. **66,** 667–674.

Breazile, J. E. (1988): The physiology of stress and its relationship to mechanisms of disease and therapeutics. Food Animal Practice **4,** 441–480.

Burny, A., Cleuter, R., Kettmann, R., Mammerickx, M., Marbaix, G., Portetelle, D., Van Den Brocke, A., Willems, L. and Thomas, R. (1988): Bovine leukaemia. Facts and hypotheses derived from the study of an infectious cancer. Vet. Microbiol. **17,** 197–218.

Fossum, C., Burny, A., Portelle, D., Mammerickx, M. and Morein, B. (1988): Detection of B and T cells, with lectins or antibodies, in healthy and bovine leukemia virus-infected cattle. Vet. Immunol. Immunopathol. **18,** 269–278.

Fujisawa, T., Abu Ghazaleh, R., Kita, H., Sanderson, C. I. and Gleich, G. I. (1990): Regulatory effect of cytokines on eosinophil degranulation. J. Immunol. **144,** 642–649.

Gennaro, R., Romeo, D., Skerlavaj, B. and Janetti, M. (1991): Neutrophil and eosinophil granules as stores of "defense" proteins. In: Harris, J. R. (ed.) Blood Cell Biochemistry, Vol. 3. Plenum Publishing Corporation, pp. 335–368.

Levkut, M., Plank, L., Levkutová, M. and Konrád, V. (1993): Pathomorphological and immunohistological changes in lymph nodes of spontaneously infected BLV cows. Acta Vet. Hung. **41,** 89–101.

Levkut, M., Plank, L., Levkutová, M. and Konrád, V. (1994): Monoclonal cytoplasmic immunoglobulin and pathomorphological reactions in lymph nodes in spontaneous BLV infection. Vet. Immunol. Immunopathol. **40,** 163–170.

Levkut, M., Soligo, D. and Ponti, W. (1991): Immunolabelling of IgM and IgG on the surface of bovine lymphocytes in the scanning electron microscope in animals naturally infected with BLV. In: Lešník, F. (ed.) Proceedings of the 4th Vet. Oncological Days in Košice, May 15–16, Košice, J.S.T.C., 1991, pp. 25–26.

Matthaeus, W. (1978): Heterogenous proprieties of BLV-precipitating immunoglobulins in bovine leucemic sera. Ann. Rech. Vet. **9,** 635–641.

Ohshima, K., Sato, S. and Okada, K. (1982): A pathologic study on initial lesions of enzootic bovine leukosis. Jpn. J. Vet. Sci. **44,** 249–257.

Pisani, H. C., Kovach, J. S., Kita, H., Seiferman, K. M., Gleich, G. I., Silver, J. E., Dennin, R. and Abrams, J. S. (1991): Administration of Interleukin-2 (IL-2) results in increased plasma concentrations of IL-5 and eosinophilia in patients with cancer. Blood **78,** 1538–1544.

Prin, L., Lequern, M. and Ameisen, J. C. (1988): HTLV-1 and malignant hypereosinophilic syndrome. Lancet **ii,** 569–570.

Samozuk, M. K., Nathwani, B. N. and Lukes, R. I. (1986): Extensive deposition of eosinophil peroxidase in Hodgkin's and Non-Hodgkin's lymphomas. Am. J. Pathol. **125,** 426–429.

Vaughan Hudson, B., Linch, D. C., Mac Intyre, E. A., Bennett, M. H., Mac Lennan, K. A., Vaughan Hudson, G. and Jellife, A. M. (1987): Selective Peripheral blood eosinophilia associated with survival advantage in Hodgkin's disease (BNLI Report No. 31). J. Clin. Pathol. **40,** 247–250.

Vukelja, S. J., Wiess, R. B., Perry, D. I. and Longo, D. L. (1988): Eosinophilia associated with adult T cell leukemia/lymphoma, Cancer **62,** 1527–1530.

Acta Veterinaria Hungarica 43 (1), pp. 153–161 (1995)

EFFECT OF MERCURY ON THE SEMINIFEROUS EPITHELIUM OF THE FOWL TESTIS

M. MARETTA[1], E. MARETTOVÁ[1], P. ŠKROBÁNEK[2] and M. LEDEČ[2]

[1]Department of Anatomy and Histology, University of Veterinary Medicine, 041 81 Košice, Komenského 73, Slovakia; [2]Poultry Research Institute, Ivanka pri Dunaji, Slovakia

(Received June 15, 1994)

Phenylmercuric chloride was applied in three doses (5 ppm, 30 ppm Hg, and 30 ppm Hg + 4 ppm Se) via the food for 60 days. The effect of Hg with an without Se was studied histologically and the data of a shortened spermatogram were evaluated. Treatment with 30 ppm Hg resulted in hypospermia, occurrence of abnormally maturing spermatozoa, reduction of the volume of semen, and decrease in the number of spermatozoa. The dose of 5 ppm Hg only resulted in the appearance of abnormally developing cells and decreased sperm motility. The addition of Se maintained spermatogenesis and the values of semen on the control level.

Key words: Mercury, selenium, cock, testis, semen

Mercury (Hg) has long been recognized as an environmental contaminant. Although Hg is distributed sparsely in nature, its hazards have increased through its widespread use (Rubinstein and Soares, 1979).

The toxic effects of mercuric chloride on a number of tissues have been reported in mammals (Berlin et al., 1969; Schroeder and Mitchener, 1975; Underwood, 1977). Accumulation of mercury has been found in the brain (Berlin et al., 1966; Chen et al., 1973), kidneys (Selye, 1970; Wisniewska et al., 1970), stomach, intestine (Nielsen and Andersen, 1989), liver (Marvan et al., 1991) and in the skin (Eyl, 1971). Less information is available on the accumulation of mercury and its activity in the testes. Burton and Meikle (1980) have studied the effect of methylmercury on the testicular function of rats, while Mohamed et al. (1987) on that of monkeys. The effect of both organic and inorganic Hg administered to male rats was studied by Vachhrajani and Chowdhury (1990). Maitra and Maitra (1989) have described the testicular changes after the administration of $HgCl_2$ to demestic fowl. The present investigation was undertaken to study the effects of organic mercury on the testes of fowl using various doses of Hg, administered with and without selenium.

0236–6290/95/$ 4.00 © 1995 *Akadémiai Kiadó, Budapest*

Materials and methods

Adult cocks (Shaver Strarcross line 579) were divided into three experimental groups of 5 birds each: group 1 received 5 ppm Hg, group 2 30 ppm Hg, group 3 30 ppm Hg + 4 ppm Se (Na_2SeO_3). One additional group was used as control. After the preparation period the birds were exposed to Hg for 8 weeks. Experimental and control birds were kept in individual laying hen cages. Mercury was used in the form of phenylmercuric chloride used as the pesticide (Agronal Super) added to feed mixture NVRM. NVRM is a complete feed mixture with methionin added for high performance laying hens in which measurements revealed 0.018 ppm of mercury. Feed and water were supplied *ad libitum*. The average individual daily uptake of the mixture was 140 g. A shortened spermatogram (three heterosperm samples from each group) was examined prior to and at two-week intervals during the application period. The volume, colour, sperm motility and number of spermatozoa were analyzed. At the end of the experiment, the testes were taken for histological examination. Tissue samples from testes were placed in 10% neutral formalin for fixation, then embedded in paraffin, sectioned and stained with haematoxylin and eosin.



Fig. 1. Seminiferous tubules from control testes. The seminiferous epithelium contains all the developing stages of germ cells. Haematoxylin and eosin (H.-E.), × 240

For ultrastructural studies, samples from testes were fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) for 2 h and postfixed in 1% OsO_4 for 1 h. The fixed tissues were dehydrated in an ascending series of ethanol and propylene oxide, then embedded in Durcupan. Thin sections for ultrastructural studies were contrasted with uranyl acetate and lead citrate, and studied with an electron microscope (Tesla BS 500). For light microscopy, 1 μm thick sections were stained with 2% toluidine blue.

Results

Histological findings

Control group. Just like other structures of the testes, the seminiferous epithelium showed no changes (Fig. 1). The seminiferous tubules contained Sertoli cells and germ cells in different phases of development from spermatogonia through spermatocytes to spermatids and mature sperm forming the various stages of the cycle of the seminiferous epithelium.

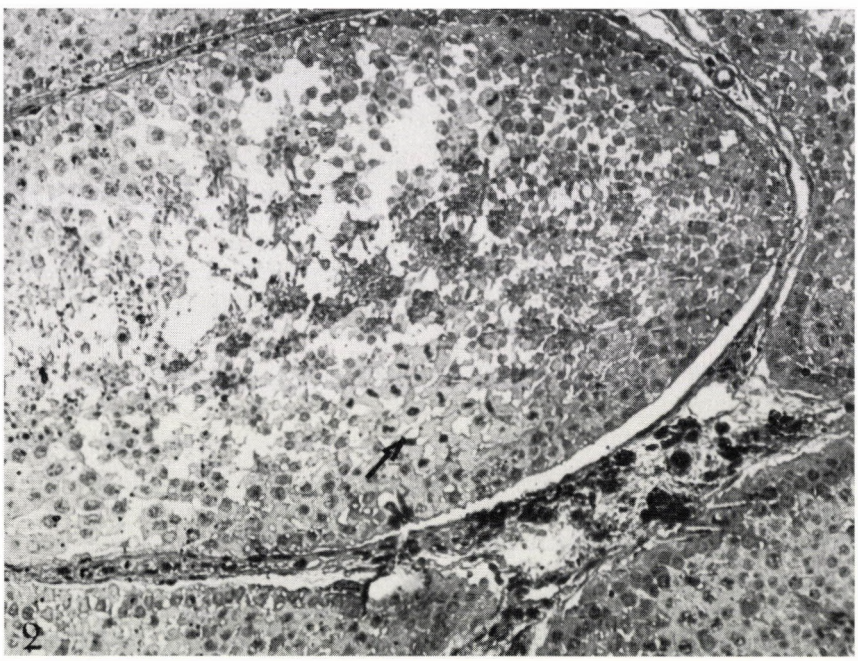


Fig. 2. Seminiferous tubules from the testes of cocks treated with 5 ppm Hg. Note the presence of abnormal spermatids (arrow) and the local disorder of germ cells. Durcupan, toluidine blue, × 280

Experimental group 1 (5 ppm Hg). No conspicuous changes were seen in this group. Partial reduction of the seminiferous epithelium, namely a reduced number of spermatids and maturing spermatozoa in some tubules, was observed. Occasionally the presence of abnormal spermatogenic elements was seen in the seminiferous tubules (Fig. 2).

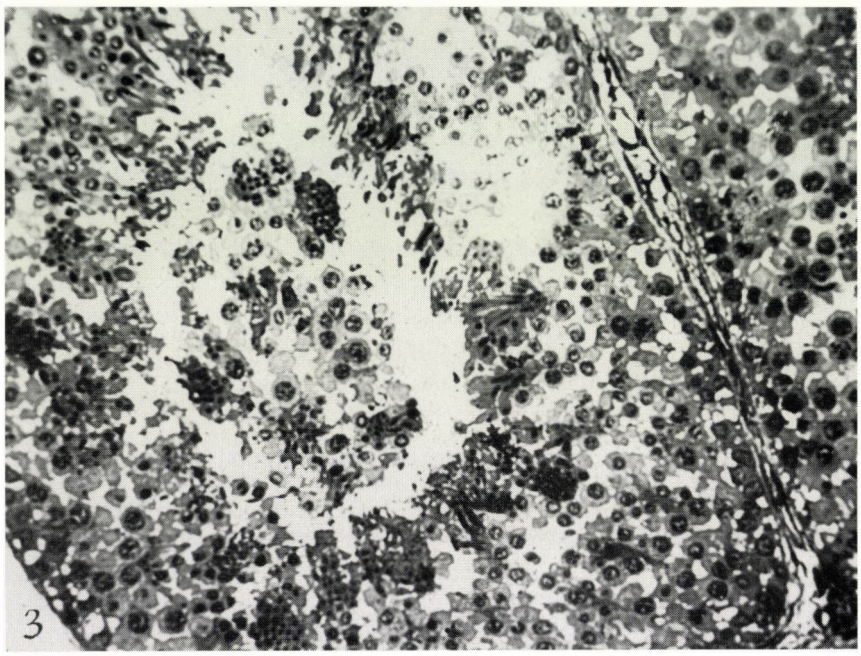


Fig. 3. Seminiferous tubules from the testes of cocks treated with 30 ppm Hg. Detachment of germ cells and the sloughing of cells at different stages of development inside the lumen are visible. Durcupan, toluidine blue, × 480

Experimental group 2 (30 ppm Hg). The superficial fibrous capsule showed marked invaginations. Increased occurrence of seminiferous tubules with a reduction in the developmental stages of sex cells, especially of maturing spermatids, was observed. Some of the seminiferous tubules revealed fragments of spermiogenic elements in the lumen; even elements of lower developmental stages could be seen (Fig. 3). An increased occurrence of abnormal spermatids and degenerating cells at later developmental stages was seen. Abnormal spermatids were located in groups inside the seminiferous epithelium. In some animals there were tubules containing fewer than normal pachytene spermatocytes. Most of the abnormal spermatids had a round, lobulated or bean-shaped nucleus. Electron microscopy (Figs 5 and 6) showed deformities in nuclear structure, acrosome shape,

location of centriolar complex and in the arrangement of mitochondries in the middle piece of the tail.

Experimental group 3 (30 ppm Hg + 4 ppm Se). In this group a certain part of the tubules showed disturbed deposition of the spermiogenic elements or release of part the sex cell population. As compared with experimental group 2, the number of maturing spermatids and spermatozoa had increased (Fig. 4).

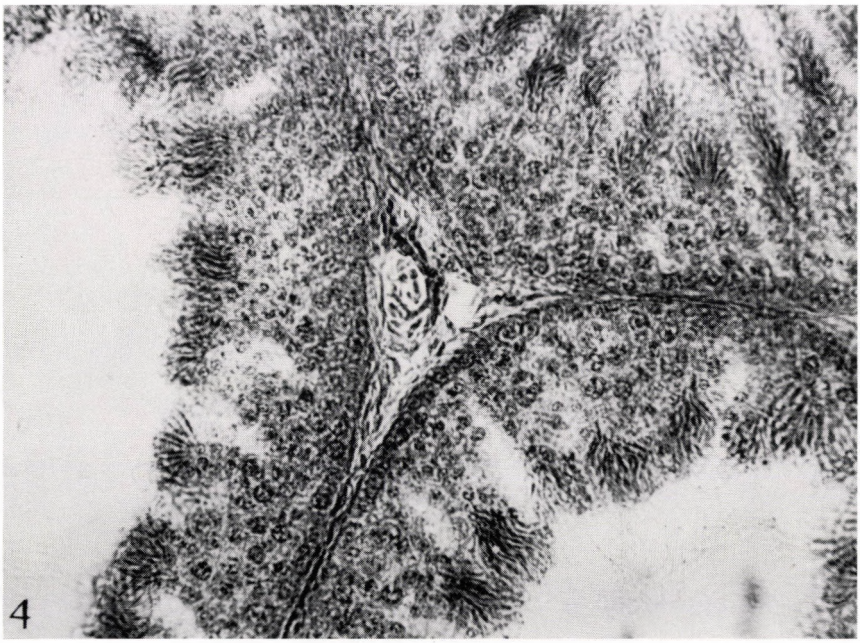


Fig. 4. Seminiferous tubules from the testes of cocks treated with 30 ppm Hg + 4 ppm Se. The seminiferous epithelium is slightly reduced but a number of developing spermatozoa can be seen. H.-E., × 480

Evaluation of semen

The examination of a shortened spermiogram revealed that the addition of 5 ppm Hg (group 1) had changed the volume of semen. On the other hand, the motility slightly decreased. In group 2 (receiving 30 ppm Hg), the volume of semen and motility of spermatozoa were maintained at the same level as in the control and in other experimental groups. The number of spermatozoa was the lowest of all groups and was lower by about 1 million that in group 1 and by 600 million less than in the control group. Experimental group 3 receiving 30 ppm Hg +

4 ppm Se showed slightly lower values than the control group in all parameters except sperm motility (Table 1).

Table 1

The effect of mercury on the value of cock semen

Group	Treatment	Mean volume ml	Total volume ml	Motility %	Number of spermatozoa in 1 mm^3
0	Control	0.34	1.36	85.00	4,281,250
1	5 ppm Hg	0.45	2.25	82.50	4,468,750
2	30 ppm Hg	0.30	1.50	86.25	3,531,250
3	30 ppm Hg + 4 ppm Se	0.26	1.30	86.25	4,062,500

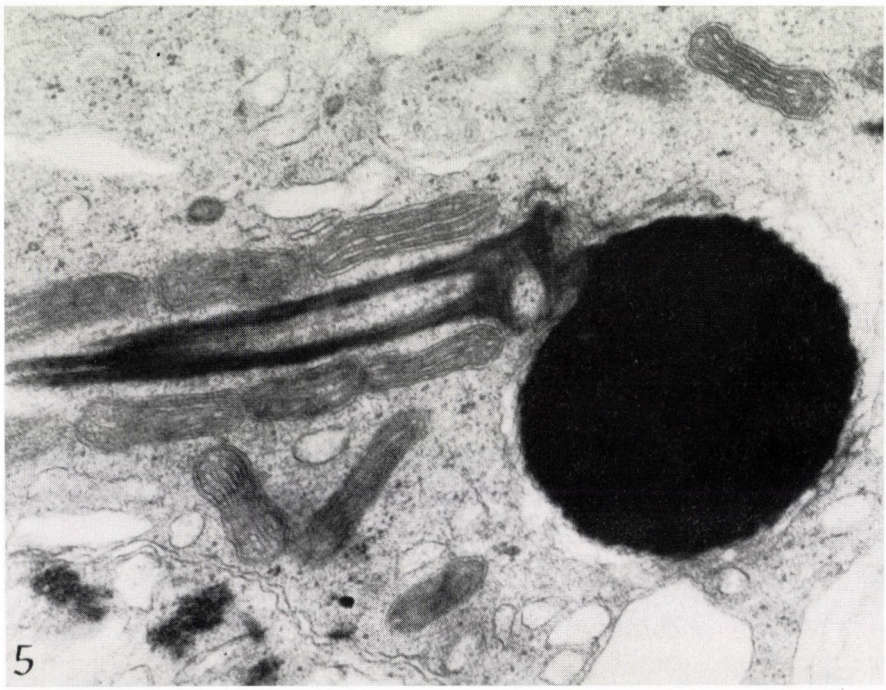


Fig. 5. Electron micrograph of the testes from cocks treated with 30 ppm Hg. Late spermatids with round nucleus and dislocation of centrioles in contact with the nucleus. × 31,500

Discussion

Histological changes in the testes as well as data from the spermiogram showed that the effects of Hg administered in the form of phenylmercury were similar to those described by Maitra and Maitra (1989) after using $HgCl_2$ and those observed by Papeseu (1978), Vogel et al. (1985) and Mohamed et al. (1987) after using alkyl- and methyl mercury compounds. The effect of mercury on testes observed in our experiment and in those of the above-mentioned authors was demonstrated by the occurrence of abnormal spermatozoa and by hypospermia.

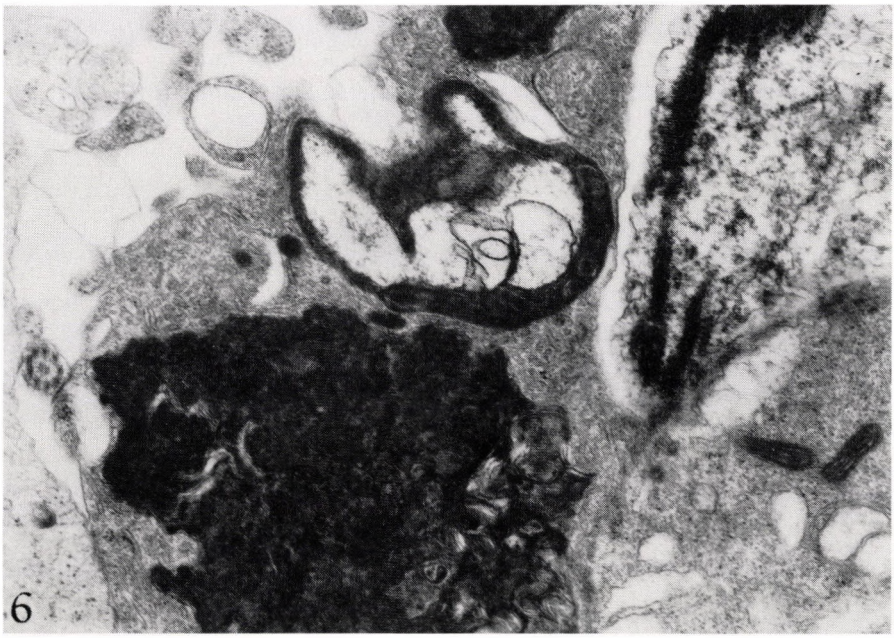


Fig. 6. Electron micrograph from the testes of cocks treated with 30 ppm Hg. Note degenerated nucleus and malformation of the acrosome. × 20,300

The protective effect of selenium described in relation to the toxic effect of Hg on other tissues (Potter and Matrone, 1974; Ganther et al., 1972) was proved in our experiment. The simultaneous addition of selenium was shown to be protective as the effect of Hg on spermatogenesis was not so adverse as that seen in the group without Se. Histologic observations showed that in the group with Se supplementation the seminiferous tubules contained a large number of later stages of developing cells and less abnormal spermatids. The positive effect of Se can be seen also in the spermatogram where the number of spermatozoa was greater in the group with than in that without Se supplementation.

The results we obtained and those of Maitra and Maitra (1989) using inorganic Hg fail to support the general opinion that organic mercury compounds are considerably more toxic to birds than their inorganic counterparts (Miller et al., 1970). It seems that this assertion is not valid for testes. The findings of Maitra and Maitra (1989) show that $HgCl_2$ causes more pronounced changes than those we found in our experiment. This difference between our results and those of Maitra and Maitra (1989) may be due either to the different mode of Hg administration or to the nature of Hg used. The protective effect of the haemotesticular barier may also play an important role in these differences and may be the source of variations between our findings and those of the above-mentioned authors. Some findings about the decrease in the nuclear diameter of Leydig cells (Maitra and Maitra, 1989) and an increase in total testicular cholesterol (Guraya, 1976) indicate that mercury in both inorganic and organic form induces changes in the endocrine system related to the process of spermatogenesis in the fowl.

References

Burton, G. V. and Meikle, A. W. (1980): Acute and chronic methylmercury poisoning impairs rat adrenal and testicular function. J. Toxicol. Environ. Health **6,** 597–606.

Berlin, M., Jersell, J. G. and von Ubisch, H. (1966): Uptake and retention of mercury in the mouse brain: A comparison of exposure to mercury vapor and intravenous injection of mercuric salt. Arch. Environ. Health **12,** 33–42.

Berlin, M., Fazackerley, J. and Nirdberg, G. (1969): The uptake of mercury in the brain of mammals exposed to mercury vapor and to mercuric salts. Arch. Environ. Health **18,** 719–729.

Chen, R. W., Ganther, H. E. and Hoekstra, W. G. (1973): Studies on the binding of methyl mercury by thionein. Biochem. Biophys. Res. Commun. **51,** 383–390.

Eyl, T. B. (1971): Organic-mercury food poisoning. N. Engl. J. Med. **284,** 706–709.

Ganther, H. E., Goudie, C., Sunde, M. L., Kopecky, M. J., Wagner, P., Sand-Hwan Oh and Hoekstra, W. G. (1972): Selenium: Relation to decreased toxicity of methylmercury added to diets containing tuna. Science **175,** 1122–1124.

Guraya, S. S. (1976): Recent advances in the morphology, histochemistry and biochemistry of steroid synthesizing cellular sites in the testes of non-mammalian vertebrates. Rev. Cytol. **47,** 99–136.

Maitra, T. and Maitra, S. K. (1989): Testicular activities in mercuric chloride treated domestic fowl. Indian J. Poult. Sci. **24,** 277–282.

Marvan, F., Samek, M., Vernerova, E. and Nemec, J. (1991): Mercury in the organs of chickens and the possibility of its elimination. Sbornik Vysoke Skoly Zemedelske v Praze, Rada B. Živočišna výroba **53,** 303–310.

Miller, V. L., Bearse, G. E. and Csonka, E. (1970): Mercury retention in several strains and stain crosses of chickens. Poultry Sci. **49,** 1101–1104.

Mohamed, M. K., Burbacher, T. M. and Mottet, N. K. (1987): Effects of methyl mercury on testicular functions in *Macaca fascicularis* monkeys. Pharmacol. Toxicol. **60,** 29–36.

Nielsen, J. B. and Andersen, O. (1989): Oral mercuric chloride exposure in mice: effects of dose on intestinal absorption and relative organ distribution. Toxicology **59,** 1–10.

Papeseu, H. I. (1978): Poisoning with alkylmercury compounds. Brit. Med. J. **1,** 1347.

Potter, S. and Matrone, G. (1974): Effect of selenite on the toxicity of dietary methyl mercury and mercuric chloride in the rat. J. Nutr. **104,** 638–647.

Rubenstein, D. A. and Soares, J. H. (1979): The effect of selenium on the biliary excretion and tissue deposition of two forms of mercury in the broiler chick, Poultry Sci. **58,** 1289–1298.

Sell, J. L. (1977): Comparative effects of selenium on metabolism of methylmercury by chickens and quail: tissue distribution and transfer into eggs. Poultry Sci. **56,** 939–948.

Selye, H. (1970): Mercury poisoning: Prevention by spironolactone. Science **169,** 775–776.

Schroeder, H. A. and Mitchener, M. (1974): Life-term effects of mercury, methyl mercury and nine other trace metals in mice. J. Nutr. **105,** 452–458.

Underwood, E. J. (1977): Trace Elements in Human and Animal Nutrition. Academic Press, New York.

Vachhrajani, K. D. and Chowdhury, A. R. (1990): Distribution of mercury and evaluation of testicular steroidogenesis in mercuric chloride and methylmercury administered rats. Indian J. Exp. Biol. **28,** 746–751.

Vogel, D. G., Margolis, R. L. and Mottet, N. K. (1985): Effects of methyl mercury binding to microtubules. Toxicol. Appl. Pharmacol. **80,** 473–486.

Wiśniewska, J. M., Trojanowska, B., Piotrowski, J. and Jakubowski, M. (1970): Binding of mercury in the rat kidney by metallothionein. Toxicol. Appl. Pharmacol. **16,** 754–763.

Acta Veterinaria Hungarica 43 (1), pp. 163–171 (1995)

VARIATIONS IN THE MILK YIELD AND MILK COMPOSITION OF DAIRY COWS DURING LACTATION

S. BEDŐ[1], Etelka NIKODÉMUSZ[1], K. PERCSICH[2] and L. BÁRDOS[3]

[1]Institute of Animal Husbandry, [2]Central Laboratory, [3]Department of Animal Physiology and Hygiene of the Gödöllő University of Agricultural Sciences, H–2103 Gödöllő, Hungary

(Received May 26, 1994)

Variations in the milk yield and milk composition of a dairy cow colony (n = 23) were analyzed during 11 months of lactation. Milk yield followed a characteristic decreasing pattern in negative correlations with solid components (milk protein, lactose, total solids, milk fat). Titrable acidity (°SH) was significantly ($P < 0.1$) higher in the milk of fresh-milking cows and it correlated negatively with lactose and positively with milk protein, milk fat and total solids. The concentrations of Zn, Fe and Cu tended to decrease, while Mn showed insignificant variation during lactation. Milk vitamin A showed a significant positive whilst milk vitamin E had a negative correlation with milk fat.

Key words: Cow, lactation, milk yield, solid components, titrable acidity, trace elements, vitamin A, vitamin E

In the past few decades safe foods, especially milk, have attracted increasing attention throughout Europe. Raw milk serves as food and also as raw material for milk products. This, together with the change-over to component pricing systems, prompts dairymen to produce milk of good quality and composition even at the expense of lowering the rate of milk production.

Milk yield and milk composition are mainly affected by genetic, nutritional and husbandry conditions. These parameters are known to vary with age, parity and through the lactation period as well.

This paper is designed to give an overall view on the variations in milk yield and composition (solid components, titrable acidity, trace elements, vitamin A, vitamin E) of a dairy cow colony during lactation. Variations in milk somatic cell count and haematologic values of this colony have been published in our previous paper (Nikodémusz et al., 1994).

0236–6290/95/$ 4.00 ©1995 *Akadémiai Kiadó, Budapest*

Table 1

Nutrient composition of feeds

Feed	(n)	Dry matter	CP	CF	DM base composition					
					Carotene	Vit. E	Cu	Mn	Fe	Zn
		g/kg	g/kg	g/kg	mg/kg					
Milking concentrate	(10)	925±18	141±15	30±7	18±9	9±7	7±3	46±10	101±17	42±16
Corn silage	(10)	381±93	60±8	18±6	27±10	17±9	5±4	43±15	172±57	30±6
Meadow hay	(4)	918±25	93±21	9±2	11±11	38±12	6±2	39±5	159±90	19±3
Alfalfa hay	(5)	850±41	134±27	12±3	15±11	22±4	6±2	24±17	84±29	28±6
Corn cuttings	(2)	458±102	62±14	15±2	32±4	26±4	5±1	55±6	157±55	23±0.1
Green alfalfa	(1)	336	152	19	19	15	4	15	79	16
Green rye	(1)	283	113	35	*	*	2	30	75	20
Grass	(1)	647	*	*	57	7	1	40	90	24

Dry matter (DM), crude protein (CP) and crude fat (CF) were determined according to the Hungarian Standard No. 6830–66.
Total carotene and vitamin E contents of feeds were determined according to Bárdos (1988).
Concentrations of trace elements were determined by atom absorption spectrophotometry.
* Not determined.

Materials and method

A colony of 23 first- and/or second-calving Holstein-Friesian and Hungarian Fleckvieh cows of the Dairy Farm of the Gödöllő University were examined from May 1992 to July 1993.

The animals were housed in a double-stalled cowshed with straw bedding. Each had access to individual feeding and water facilities. The basic feed ration comprised a milking concentrate and dry fodder (alfalfa or meadow hay). This diet was supplemented with corn silage from October to August and green fodder (green rye, alfalfa, corn cuttings, grass) from mid-May to late-September. Dry matter (DM) base composition of feeds is given in Table 1.

Cows were milked twice daily between 05:00 and 06:00 a.m. and between 16:00 and 17:00 p.m. Individual milk yield was checked once a month. Milk composition was determined on composite p.m.-a.m. samples taken at monthly intervals.

Solid components including milk fat (MF), milk protein (MP), lactose (LC) and total solids (TS) were determined with a MILKO-SCAN 133 B device (L. Foss Electric, Denmark).

Acidity of milk (°SH) was measured by titrimetry according to the Hungarian Standard No. 3707–65 using an automated burette.

The concentrations of trace elements (Cu, Mn, Fe, Zn) in milk were measured from ash (dissolved in 6.5 % HNO_3) by atom absorption spectrophotometry.

Milk vitamin A and vitamin E were determined by colorimetry according to Bárdos (1988) and Bieri (1964), respectively, using a SPECOL spectrophotometer.

Data were assigned into 11 lunar months of lactation (1 lunar month = 28 days). Means ± S.D. values were compared by Student's *t*-test (Sváb, 1973). Correlation analysis of data was performed by simple regression as outlined in STATGRAPHICS (STSC Inc. and Statistical Graphics Corp., 1985).

Results

A lactation curve characteristic of the dairy cow colony is shown in Fig. 1. (A milk yield that commenced with 23 litres, decreased gradually to 18–19 litres in months V–VI, then to 11 litres in month XI.)

Variations in solid components and °SH of milk are given in Fig. 2.

After colostral peaks in weeks 1–2 MF, MP and, thereby, TS were decreasing till month IV then again tended to increase. Lactose increased slightly by weeks 3–4; after that it varied little (ca. 4.8–5.1%). Milk acidity was significantly ($P < 0.01$; $P < 0.05$) higher in weeks 1–2 (°SH = 10.33), compared to the rest of

lactation (°SH = 6.50–7.42). The between-month differences of the variables were insignificant.

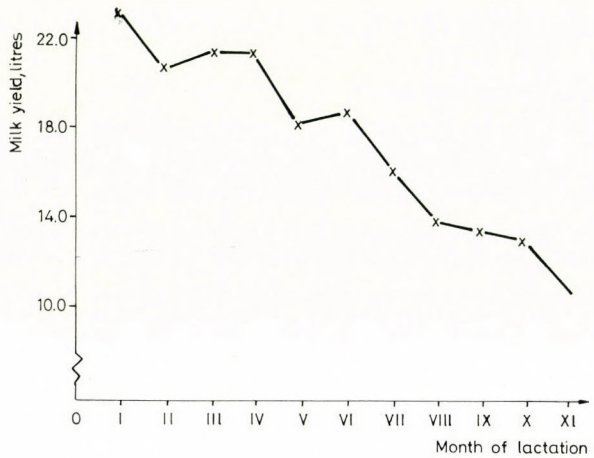


Fig. 1. Lactation curve of dairy cows (overall S.D. = ± 5.3)

Table 2

Variations in milk concentrations of trace elements (Mean ± S.D.)

Lactation month	(n)	Cu	Mn	Fe	Zn
		mg/litre			
I	(17)	0.106±0.066	0.043±0.017	0.87±0.91	3.38±1.44
II	(16)	0.094±0.016	0.035±0.008	0.70±0.27	2.52±0.88
III	(16)	0.079±0.026	0.039±0.017	0.73±0.67	2.29±0.58
IV	(17)	0.078±0.039	0.044±0.018	0.71±0.39	2.37±0.56
V	(19)	0.091±0.075	0.038±0.014	0.58±0.23	2.31±0.87
VI	(19)	0.065±0.045	0.043±0.025	0.67±0.55	2.34±0.71
VII	(18)	0.088±0.085	0.039±0.016	0.63±0.59	2.62±0.57
VIII	(18)	0.061±0.037	0.047±0.018	0.79±0.76	2.62±0.61
IX	(19)	0.065±0.057	0.048±0.029	0.67±0.43	2.41±0.83
X	(17)	0.070±0.043	0.045±0.017	0.66±0.78	2.67±0.62
XI	(16)	0.074±0.064	0.049±0.019	0.64±0.41	2.46±0.60

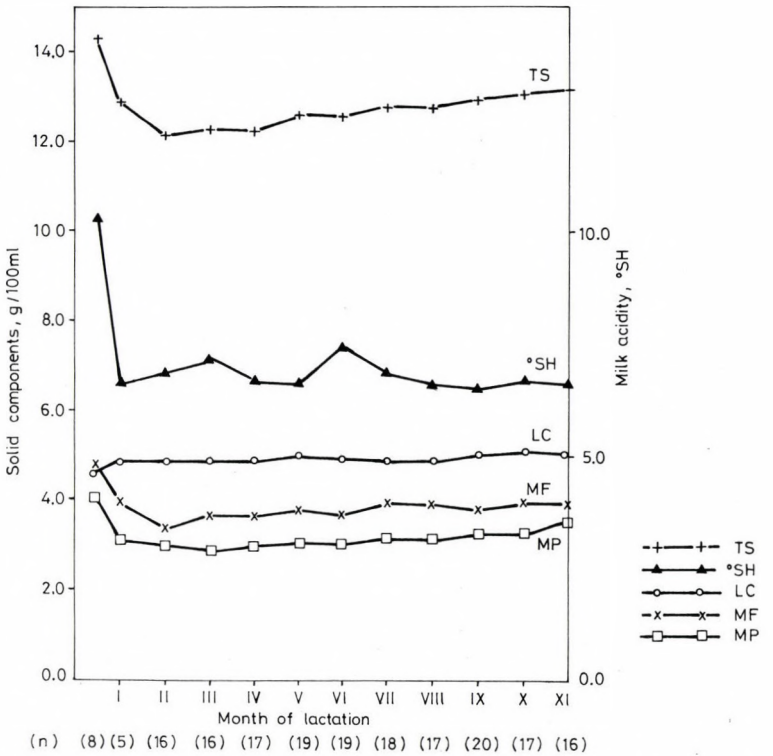


Fig. 2. Variations in solid components and acidity of milk during lactation. TS = total solids, overall S.D. = 1.18; °SH = milk acidity, overall S.D. = 1.40; LC = lactose, overall S.D. = 0.27; MF = milk fat, overall S.D. = 1.08; MP = milk protein, overall S.D. = 0.45

Milk concentrations of all trace elements but Mn tended to decrease during lactation (Table 2). The decrease in Zn and Cu was significant ($P < 0.1$; $P < 0.05$) through months II–XI and in months V and VIII–XI, respectively.

Variations in the milk levels of vitamin A and vitamin E are presented in Fig. 3.

Vitamin A, highest in month I (2.32 mg/litre), dropped by ca. 42% ($P < 0.05$) in month II, then it showed no consistent increases. Vitamin E increased gradually (from ca. 1.1 to 1.4 mg/litre) till month IX, then it dropped below the initial value. The changes in milk vitamin E were significant at a level of $P < 0.01$ and $P < 0.05$, respectively.

Correlation analysis of the data (Table 3) revealed negative correlations between milk yield (MY) and solid variables (MP, LC, TS, MF), between lactose (LC) and MF, between LC and °SH, between LC and MP, and between MF and vitamin E. Positive correlations were found between MF and MP, between MP

and °SH, between MF and °SH, between TS and °SH, and between MF and vitamin A. The correlations were mostly significant or nearly significant.

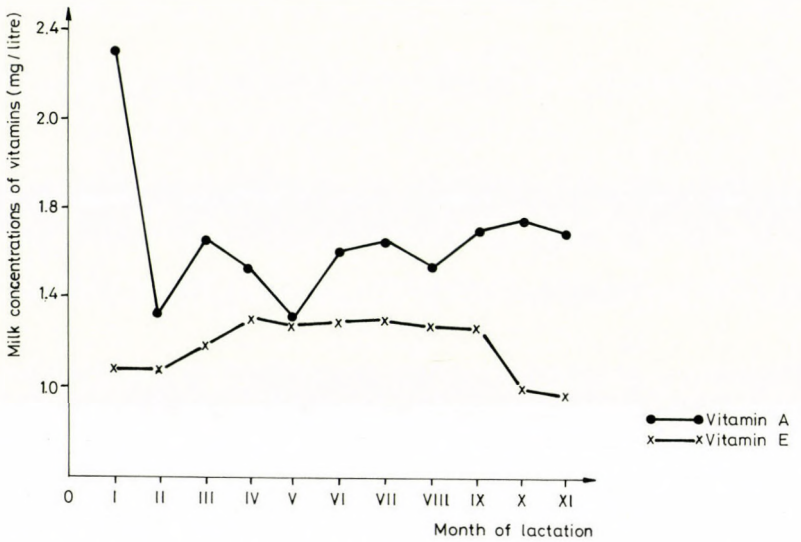


Fig. 3. Variations in milk concentrations of vitamins A and E during lactation (overall S.D. for vitamin A = ± 0.87, for vitamin E = ± 0.45)

Table 3

Correlations observed among the variables

Variable	Total sample (n=192)		Monthly means (n=11)	
	r	P	r	P
MY – TS	–0.19	< 0.01	–0.41	> 0.10
MY – MP	–0.32	< 0.001	–0.45	> 0.10
MY –LC	–0.17	< 0.05	–0.71	< 0.05
MY –MF	–0.03	>0.10	–0.04	> 0.10
TS – °SH	0.22	< 0.01	0.35	> 0.10
MF – °SH	0.18	< 0.05	0.58	< 0.05
LC – °SH	–0.23	< 0.01	–0.66	< 0.05
MP – °SH	0.27	< 0.001	0.29	> 0.10
MF – MP	0.14	< 0.1	0.74	< 0.01
MF – LC	–0.26	< 0.001	–0.38	> 0.10
MP – LC	–0.20	< 0.01	0.03	> 0.10
MF – Vit. A	0.29	< 0.001	0.84	< 0.001
MF – Vit. E	–0.008	> 0.1	–0.18	> 0.10

Discussion

The lactation curve presented for the first- and/or second-calving cows of the Holstein-Friesian and Hungarian Fleckvieh breeds is similar to that reported for 75 Hungarian Fleckvieh × Holstein-Friesian cows (Székely, 1993). The variations observed in milk composition of our colony during lactation are also comparable to previous data.

The colostrum, as a feed of full value for baby calves, is rich in nutrients, minerals, vitamins, as well as blood cells and epithelial cells, i.e. somatic cells, and immunoglobulins (Csiszár, 1954; Szentpéteri et al., 1986, Bicsérdi and Süle, 1991).

After the colostral peaks milk fat, milk protein and TS decrease through months I–III, then increase gradually parallel to the decrease in milk yield (Csapó and Csapó, 1983; Székely, 1993). The significant negative correlations between milk yield and the solid variables together with the significant positive correlation between milk fat and milk protein (Table 3) also support this pattern.

In good agreement with previous studies, lactose appeared relatively stable due to its buffering between the osmotic pressure of milk and that of alveolar fluid and blood plasma, respectively (Csapó, 1990).

The colostral peak and the subsequent variation observed in °SH are considered normal. Raw milk is characterized by an °SH of 6.2–7.0, which may rise to 8.5–11.5 in colostrum and drop below 6.0 in samples of old milking cows (Csiszár, 1954; Hungarian Raw Milk Standard No. 17, 1991). Old milking cows in our colony (n=9) also exhibited a mean of 6.0 for °SH. The actual and potential titrable acidity may vary with concentrations of acidic phosphates, citrates, bicarbonates, carbonic acid, amino acids, proteins and lactic acid present in milk (Csiszár, 1954). This is confirmed by the positive correlation between TS and °SH and between milk protein and °SH, as well as by the negative correlation between lactose and °SH (Table 3).

In our samples Zn, Cu and Fe tended to decrease, Mn was relatively stable in milk during lactation, contrary to the increasing patterns reported for the three latter in the Hungarian Fleckvieh, Holstein-Friesian and Hungarian Fleckvieh × Holstein-Friesian cows fed mainly on grass (Csapó and Csapó, 1982). Concentrations of Zn, Cu and Mn were also lower in milk samples of our cows receiving conventional ration-fodder diets.

Milk vitamin A varied parallel and in significant positive correlation with milk fat. In contrast, milk vitamin E showed a non-significant and negative correlation with milk fat (Fig. 3, Table 3). The difference may partly be attributed to a higher supplementation of vitamin A in the milking concentrate, compared to vitamin E (13,500,000 vs. 9,000 IU according to the manufacturing company). Cows supplemented with 170,000 IU of vitamin A produced significantly more

4% fat corrected milk (Oldham et al., 1991). The values presented here for milk vitamin A and vitamin E were comparable to data reported in the literature. Namely, the colostrum of Jersey cows contains approximately 4.3 mg vitamin A/litre (Johnston and Chew, 1984) and 1.91 mg vitamin E/litre (Hidiroglou, 1989) as determined by colorimetry and HPLC, respectively. In fresh milk vitamin A and vitamin E may amount to 0.2–2 and 1–2 mg/litre, respectively (Balatoni and Ketting, 1981).

In spite of small sample size, the variations presented for the milk constituents and properties reflect more the lactation pattern than the season. Only 38% of the total sample (n=192) represented the summer surplus "carotene" season (from mid-May to late-September). Dietary beta carotene has no significant effects on milk production or milk components (Rakes et al., 1985). Vitamin A may be more effective (Oldham et al., 1991; Hullár and Brand, 1993) and it was provided in the milking concentrate of cows at a standard level throughout the year.

References

Balatoni, M. and Ketting, F. (1991): Handbook of Milk Industry (in Hungarian). Mezőgazdasági Könyvkiadó, Budapest.

Bárdos, L. (1988): Measurement of components of vitamin A level (retinol, retinyl-ester) and total carotene content in biological fluids (in Hungarian, with English abstract). Magyar Állatorvosok Lapja **43,** 113–116.

Bicsérdi, Gy. and Süle, J. (1991): Food Hygiene I. Milk hygiene (in Hungarian). Faculty of Animal Physiology and Hygiene, Univ. Agric. Sci. Debrecen, Hódmezővásárhely.

Bieri, J. G. (1964): Serum vitamin E levels in normal adult population in Washington D.C. area. Proc. Soc. Exp. Biol. Med. **117,** 131–133.

Csapó, J. (1990): Factors affecting the protein content of milk (in Hungarian). Tejipar **51,** 91–98.

Csapó, J. and Csapó, Zs. (1982): Examination of the mineral content in milk of Hungarian Fleckvieh and Holstein-Friesian cows and of their crossbreds during lactation (in Hungarian). Tejipar **31,** 55–60.

Csapó, J. and Csapó, Zs. (1983): Examination of protein content in milk of Hungarian Fleckvieh, Holstein-Friesian and Hungarian Fleckvieh × Holstein-Friesian F1 cows during lactation (in Hungarian). Tejipar **32,** 39–46.

Csiszár, V. (1954): Hygienics of Milk Production (in Hungarian). Mezőgazdasági Kiadó, Budapest. pp. 14–28, 49–51.

Hidiroglou, M. (1989): Mammary transfer of vitamin E in dairy cows. J. Dairy Sci. **72,** 1067–1071.

Hullár, I. and Brand, A. (1993): Nutritional factors affecting milk quality, with especial regard to milk protein: A review. Acta Vet. Hung. **41,** 11–32.

Johnston, L. A. and Chew, B. P. (1984): Peripartum changes of plasma and milk vitamin A and β-carotene among dairy cows with or without mastitis. J. Dairy Sci. **67,** 1832–1840.

Nikodémusz, E., Bedő, S., Pichler, A. and Szép, P. (1994): Variations in milk somatic cell count and haematologic values of dairy cows during lactation. Acta Vet. Hung. **42,** 131–139.

Oldham, E. R., Eberharthy, R. J. and Muller, L. D. (1991): Effects of supplemental vitamin A or beta carotene during the dry period and early lactation on udder health. J. Dairy Sci. **74,** 3775–3781.

Rakes, A. H., Owens, M. P., Britt, J. H. and Whitlow, L. W. (1985): Effects of adding beta-carotine to rations of lactating cows consuming different forages. J. Dairy Sci. **68,** 1732–1737.

STST Inc. and Statistical Graphics Corp. (1985): STATGRAPHICS Statistical Graphics System. Version 4.0. Licensed Software, USA.

Sváb, J. (1973): Biometrical Methods in Research (in Hungarian). Mezőgazdasági Kiadó, Budapest.

Székely, Zs. (1993): Variations in milk composition and somatic cell count during lactation (in Hungarian). Thesis, Gödöllő Univ. Agric. Sci., Gödöllő. pp. 49–58.

Szentpéteri, J., Csapó, J., Csapó, Zs., Karle, G. and Gundel, K. (1986): Effect of criss-crossing of Jerseys and Holstein Friesians on the basis of Hungarofriesian cattle on the composition of the colostrum and milk (in Hungarian, with English abstract). Állattenyésztés és Takarmányozás **35,** 549–558.

Acta Veterinaria Hungarica 43 (1), pp. 173–177 (1995)

EFFECT OF SEVERE ENERGY RESTRICTION AND REFEEDING ON THYROID HORMONES IN BULLS

J. JANAN[1], P. RUDAS[1*], T. BARTHA[1], S. BOZÓ[2] and Gy. GÁBOR[2]

[1]Department of Physiology and Biochemistry, University of Veterinary Science, H–1400 Budapest, P.O. Box 2, Hungary; [2]Research Institute for Animal Breeding and Nutrition, H–2103 Herceghalom, Hungary

(Received May 20, 1994)

Fifty-three Holstein-Friesian breeding bulls (944.99 ± 14.59 kg) were fasted for 4 weeks. The influence of feeding on thyroid hormones was studied by comparing a starting point with a 4-week fasting period and a refeeding period. Blood samples were taken via a jugular vein catheter at 8:00 a.m. one day before, then once every week during, and two times after the fasting period. Plasma thyroxine (T_4) and triiodothyronine (T_3) levels were determined by direct radioimmuno-assay. The concentration of T_4 and T_3 decreased during fasting. The concentration of T_3 increased after refeeding, but that of T_4 did not. These data suggest that fasting is associated with a decrease in the peripheral conversion of T_4 to T_3 and, consequently, less T_4 is converted into T_3.

Key words: Bull, fasting, refeeding, serum thyroxine, triiodothyronine

Thyroid hormones play an important role in metabolic processes. The interaction between thyroid hormones and energy intake in bulls is of interest. Previous experiments have shown a marked decrease in the plasma concentration of triiodothyronine in mammals during starvation (Pethes et al., 1985). In chickens, it is evident that feed restriction lowers the concentration of circulating triiodothyronine probably by inhibiting the activity of liver deiodinase, and also decreases the sensivity of the pituitary–thyroid axis (Bartha et al., 1989). The secretion of hormones from the thyroid gland was strongly reduced during starvation in bulls (Tveit and Almida, 1980). Tveit and Larsen (1983) reported that the secretion of hormones is nearly stopped during starvation in bull calves.

In the present experiment the influence of feeding on thyroid hormones was studied by comparing a starting point with a 4-week fasting period and a refeeding period.

* To whom reprint requests should be addressed

0236–6290/95/$ 4.00 © 1995 *Akadémiai Kiadó, Budapest*

Materials and methods

Fifty-three Holstein-Friesian breeding bulls were used in the experiment. The following procedure was applied:

(1) Fasting: (1/a) on day 1, introductory phase with 10% experimental diet (see later) + 90% original (see later). (1/b) adaptation on days 2–4: 30–80% of experimental diet. (1/c) from day 5 for 4 weeks: 100% of experimental diet. (2) Refeeding: without adjustment period.

Apart from the diets the bulls were fed high-quality barley straw *ad libitum*. The experimental diet contained 3.9 MJ NEm and 216 g crude protein. This resulted in very low undernutrition. The nutrients of barley straw fed *ad libitum* did not disturb starvation.

Blood samples were taken via a jugular vein catheter at 8:00 a.m. one day before, then once every week during, and twice after the fasting period into heparinized tubes. The blood samples were cooled down immediately after collection in the laboratory they were centrifuged at 3000 rpm to harvest the plasma, which was then frozen at –20 °C. The plasma thyroxine and triiodothyronine levels were determined by direct radioimmunoassay (Pethes et al., 1978).

Results

Table 1 shows the mean T_4 and T_3 values of all the animals during the control, fasting and refeeding periods.

Table 1

Concentration of thyroxine (T_4) and triiodothyronine (T_3) in the blood serum of breeding bulls before, during and after feed restriction (ng/ml)

T_4	121.14	110.6	92.1	76.94	82.015	81.19	73.9
SEM	6.85	6.03	4.23	3.69	4.63	3.46	3.37
n	52	52	53	52	53	52	53
T_3	0.29	0.27	0.21	0.21	0.26	0.38	0.62
SEM	0.02	0.024	0.08	0.08	0.02	0.04	0.04
n	53	53	53	53	53	53	53

As shown in Table 1 and Figs 1 and 2, the concentration of T_4 and T_3 decreased during fasting and T_3 increased after refeeding. No increase in T_4 was demonstrable after refeeding as shown in Fig. 1.

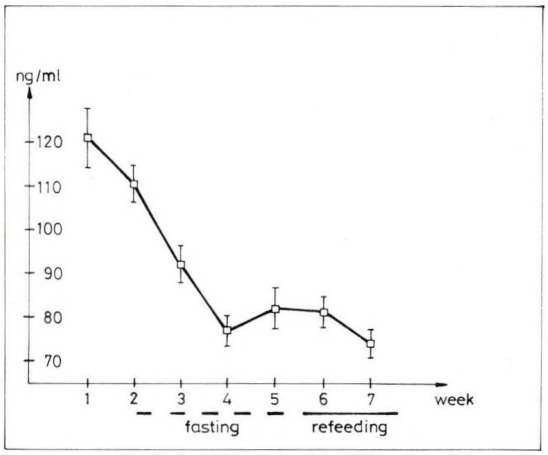


Fig. 1. Serum T_4 concentration of bulls before, during and after feed restriction

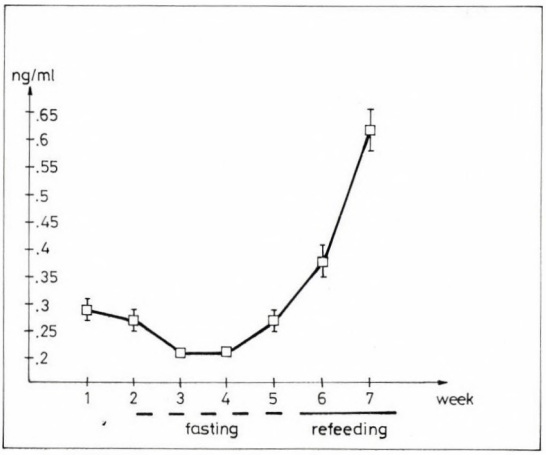


Fig. 2. Serum T_3 concentration of bulls before, during and after feed restriction

Discussion

Thyroid gland function and thyroid metabolism are under the influence of several physiological and environmental factors. Our results indicate that the concentration of T_4 and T_3 decreased during fasting. Fasting ruminants are characterized metabolically by hypoglycaemia, hyperketonaemia (Emmanuel and Kennelly, 1984) and hyperlipidaemia (DiMarco et al., 1981). The response of thyroid economy to fasting depends on several factors. It should be noted that the serum con-

centration of a particular iodinated compound depends not only upon its production but also upon its affinity to carrier proteins, its tissue distribution, its rate of degradation and, finally, its clearance. It is well known that humans survive prolonged fasting by using stored fat as a source of energy.The key hormones responsible for the ability to shift to fat as a source of energy are epinephrine and the thyroid hormones. More than 30% of the extrathyroidal body pool of T_3 is derived from the peripheral monodeiodination of T_4 (Sterling et al., 1970). In the centre of this mechanism stands the deiodinase enzyme that can convert thyroxine produced by the thyroid gland either to active triiodothyronine or to inactive reverse-triiodothyronine, depending on the actual needs of the organism (Silva and Larsen, 1985). The results of Kahl et al. (1984) show the presence of a very active enzymatic system responsible for the peripheral 5'-monodeiodination of T_4 to T_3 in cattle. Among a series of factors that might influence this system is the availability of energy equivalents to the cells. Therefore the response of thyroid hormone metabolism to fasting may assume the form of changes in the secretion rate of the central thyroid product (T_4 and T_3) or alteration of the peripheral deiodination of T_4 or of the utilization of T_3 on cellular level. In accordance with the findings of Blum et al. (1985), our results show that in reduced food intake the concentrations of T_4 and T_3 are reduced. Caloric deprivation in sheep also led to decreased T_3 levels and overnutrition to increased T_3 (Blum et al., 1980). During the 4-week fasting used in this experiment the concentration of T_4 decreased at a rate of 50–70%, indicating that little T_4 secretion was taking place. T_3 also decreased after fasting. As a portion of T_3 is produced in the thyroid gland, our results may suggest that the secretion of hormones from the thyroid gland is decreased during starvation. As about 70% of T_3 is known to derive from T_4 in the peripheral tissues, we can also suppose that deiodinase activity almost completely ceases after fasting. Liver deiodination activity is the first to react to energy restriction (Bartha, 1993). After a certain period of time the activity of the enzyme returns to normal. Since blood was taken one week after the beginning of restricted feeding, deiodinase activity may be assumed to have returned to the normal level.

Refeeding caused a rapid increase in T_3, but the concentration of T_4 did not change. This is contrary to results from young bulls in which refeeding caused a rapid increase in T_3 and T_4 (Tveit and Larsen, 1983). A possible explanation for this is the higher activity of deiodinase enzyme, which converts more T_4 to T_3. Consequently the concentration of T3 rose and that of T_4 remained unchanged. Blum et al. (1985) also reported that in steers the concentration of T_4 and T_3 increased within days in response to refeeding. According to Rudas and Newcomer (1987), food intake in fasted animals causes a prompt release of enteroglucagon which stimulates insulin secretion even above the fasting level. This might directly or indirectly increase the activation of thyroid hormones which then assists in raising the energy yield from the substrates available for the cells.

We can conclude from these studies that fasting decreases the serum concentration of T_4 and T_3 as it is associated with a decrease in the peripheral conversion of T_4 to T_3 and, consequently, less T_4 is converted into T_3. The concentration of T_3 increased after refeeding but there was no change in T_4 concentration.

Acknowledgements

We would like to express our gratitude Mrs. Elisabeth Kraiss for her expert technical assistance and to Mrs. Mária Nádas for her excellent help. This work was supported in part by a grant from the National Research Fund of Hungary (OTKA), no. 2429 and no. 1259.

References

Bartha, T. (1993): Thyroid hormone metabolism in broiler chickens as influenced by exogenous and endogenous factors. Dissertation de agricultura. Doctoraatsproefschrift Nr. 233 aan de Faculteit der Landbouwwetenschappen van de K. U. Leuven.

Bartha, T., Rudas, P., Fekete, S. and Pethes, G. (1989): Restricted feed intake influences thyroid hormone production and peripheral deiodination in chickens. Acta Vet. Hung. **37,** 241–246.

Blum, J. W., Gingins, M., Vitins, P. and Bickel, H. (1980): Thyroid hormone levels related to energy and nitrogen balance during weight loss and regain in adult sheep. Acta Endocrinol. **93,** 440–447.

Blum, J. W., Schnyder, W., Kunz, P. L., Blom, A. K., Bickel, H. and Schurch, A. (1985): Reduced and compensatory growth: endocrine and metabolic changes during food restriction and refeeding in steers. J. Nutr. **115,** 417–424.

DiMarco, N. M., Beitz, D. C. and Whitehurst, G. B. (1981): Effect of fasting on free fatty acid, glycerol and cholesterol concentration in blood plasma and lipoprotein lipase activity in adipose tissue of cattle. J. Animal Sci. **52,** 75–82.

Emmanuel, B. and Kennelly, J. J. (1984): Effects of propionic acid on ketogenesis in lacting sheep fed restricted rations or deprived of food. J. Dairy Sci. **67,** 344–350.

Kahl, S., Bitman, J. and Rumsey, T. S. (1984): Extrathyroidal thyroxine-5'-monodeiodinase activity in cattle. Domestic Animal Endocrinology **1,** 279–290.

Pethes, G., Bokori, J., Rudas, P., Frenyó, V. L. and Fekete, S. (1985): Thyroxine, triiodothyronine, reverse-triiodothyronine, and other physiological characteristics of periparturient cows fed restricted energy. J. Dairy Sci. **68,** 1148–1154.

Pethes, G., Losoncy, S. and Rudas, P. (1978): Measurement of serum triiodothyronine by radioimmunoassay (in Hungarian, with English abstract). Magyar Állatorvosok Lapja **33,** 177–182.

Rudas, P. and Newcomer, W. S. (1987): Glucagon, insulin and the thyroid. Acta Vet. Hung. **35,** 297–305.

Silva, E. J. and Larsen, P. R. (1985): The potential of brown adipose tissue type II thyroxine 5'-deiodinase as a local and systematic source of triiodothyronine in rats. J. Clin. Invest. **76,** 21–30.

Sterling, K., Brenner, M. A. and Neuman, E. S. (1970): Conversion of thyroxine to triiodothironine in normal human subjects. Science **169,** 1099–1100.

Tveit, B. and Almida, T. (1980): T4 degradation rate and plasma levels of TSH and thyroid hormones in ten young bulls during feeding condition and 48 hours of starvation. Acta Endocrinol. **93,** 435–439.

Tveit, B. and Larsen, F. (1983): Suppression and stimulation of TSH and thyroid hormones in bulls during starvation and refeeding. Acta Endocrinol. **103** (2), 223–226.

Acta Veterinaria Hungarica 43 (1), pp. 179–190 (1995)

EFFECT OF HYDROTHERMAL TREATMENT OF RICE STRAW ON ITS COMPOSITION AND *IN SACCO* DIGESTIBILITY AND *IN VITRO* FERMENTATION BY RUMEN MICROORGANISMS

M. ADYA, V. K. SAREEN and SUDARSHAN SINGH*

Department of Biochemistry, Punjab Agricultural University, Ludhiana 141004, India

(Received June 15, 1994)

Chemical composition, *in sacco* rumen disappearance of various cell wall constituents (CWC) and *in vitro* fermentation pattern of hydrothermally treated (1 to 14 kp/cm^2 pressure for 5 min) rice straw was examined. At 10 kp/cm^2 pressure treatment (maximum effect) the contents of dry matter (DM), organic matter (OM), neutral detergent fibre (NDF), acid detergent fibre (ADF), hemicellulose (HC) and cellulose (CE) were decreased by 32.5, 35.3, 27.8, 10.2, 61.2 and 25.1%, respectively ($P < 0.05$), over the untreated control. The *in sacco* rumen disappearance of DM, OM, HC and CE from rice straw treated at 8 kp/cm^2 pressure (maximum effect) and incubated for 48 h was increased from 53.2 to 77.7, 52.4 to 80.3, 49.5 to 82.0 and 49.2 to 79.3%, respectively ($P < 0.01$). *In vitro* production of total volatile fatty acids and the content of TCA-insoluble protein was also significantly higher ($P < 0.05$) on treated compared with untreated straw.

Key words: Rice straw, hydrothermal treatment, rumen digestibilities, ruminants, buffalo

Low grade roughages/crop residues will continue to serve as important feed resources for ruminants. These feeds, however, do not produce sufficient energy to support an optimal level of growth and lactation because of resistance of the fibre they contain to digestion by rumen microorganisms. Pretreatment with alkali, ammonia or oxidizing agents has been used for improving their nutritive values. However, the cost and availability of such chemicals may limit their use in the future. Limited studies have been done on the hydrothermal treatment of straws/crop residues (Oji and Mowat, 1978, 1979; Hart et al., 1980; Rangnekar et al., 1982; Rai and Mudgal, 1986; Okamoto and Hidenori, 1989) to improve their nutritional value. This investigation examines the effect of hydrothermal treatment of rice straw on its composition and *in sacco* digestibility and *in vitro* fermentation by rumen microorganisms in buffalo calves.

* Address reprint requests to Dr. Sudarshan Singh, Professor of Biochemistry

0236–6290/95/$ 4.00 © 1995 *Akadémiai Kiadó, Budapest*

Materials and methods

Twelve g rice straw (passed through an 1-mm screen) taken in triplicate was soaked in water (1:10, w/v) in individual flasks and autoclaved (VEB Autoklav, Leipzig, GDR) at different pressures for 5 min. The contents of each flask were then filtered, the specific volume of filtrate determined, and the residue dried at 50 °C in an oven. The dried residue and filtrate of one flask were divided into 12 equal parts and each part of residue and filtrate taken together was used for *in vitro* fermentation studies. The residue from each of the other 2 flasks was divided into 4 equal parts and used for determination of its composition and for *in sacco* disappearance studies.

Two rumen-fistulated buffalo calves (*Bubalus bubalis*) of 170 kg average body mass were fed daily a growth ration of 5 kg wheat straw and 2 kg concentrates. The animals were kept on this ration for one month before used for *in sacco* disappearance and *in vitro* rumen fermentation experiments.

Dacron bags of 16 × 7 cm size made from old parachute cloth (pore size 70 μm) were used for *in sacco* disappearance of dry matter (DM) and cell wall constituents (CWC). The treatment of bags and the rumen incubation procedure were the same as described by Mehrez and Orskov (1977). The bags containing either 3 g untreated ground rice straw (1-mm screen) or residue of 3 g hydrothermally treated rice straw were inserted into the rumen of buffalo calves and the disappearance of DM and CWC was examined after 24 and 48 h of incubation. For calculating the disappearance from treated straw the weight of residue of treated straw taken in the bag was also considered as 3 g. Neutral detergent fibre (NDF), acid detergent fibre (ADF) and cellulose (CE) in the untreated and treated rice straw and residue left after their *in sacco* incubations were estimated by the procedure of Goering and Van Soest (1970). Hemicellulose (HC) was calculated as the difference between NDF and ADF.

For *in vitro* rumen fermentation studies, samples of rumen fluid were obtained before feeding the calves in the morning by suction through a perforated plastic tube. The samples from both calves were pooled on an equal volume basis and strained through 2 layers of muslin cloth in prewarmed thermos. Substrates (1 g ground rice straw as well as 1/12 part of residue and filtrate from one autoclaved flask taken together) were dispersed in 25 ml phosphate buffer (same as buffer II used by Sharma et al., 1981) of ph 6.8 and saturated with nitrogen gas taken in conical flasks fitted with stopper and bunsen valve (Johnson, 1966), and incubated at 39 °C in an atmosphere of CO_2 for 20 min. The strained rumen fluid (25 ml, saturated with CO_2) was then added in each flask and the contents were gassed with CO_2 again; they were stoppered and incubated as above for 2–12 h. All the incubations were done in duplicate at 39 °C in a temperature-controlled water bath with shaking platform. Zero-hour controls were also run simultane-

ously. At the end of the incubation period, fermentation was stopped in each flask by adding 1 ml saturated mercuric chloride solution. NH_3-N and total volatile fatty acids (TVFA) in the incubation mixture (IM) were measured using the methods of Weatherburn (1967) and Barnett and Reid (1957), respectively. Proteins were precipitated from the IM with 10% TCA, dissolved in 0.1 N NaOH and estimated (Lowry et al., 1951). Empirical analysis of the data was done by analysis of variance and linear and log-linear regression.

Results

Hydrothermal treatment of rice straw significantly ($P < 0.05$) decreased the contents of DM, OM, NDF, HC and CE and the effect was more pronounced up to 10 kp/cm^2 pressure. The content of ADF was, however, decreased significantly ($P < 0.05$) only up to 6 kp/cm^2 pressure. At 10 kp/cm^2 pressure treatment, the contents of DM, OM, NDF, ADF, HC and CE were decreased by 32.5, 35.3, 27.8, 10.2, 61.2 and 25.1%, respectively, over the untreated control (Table 1). The data generated by the applied linear regression model (Table 2) showed that the extent of variation in the contents of DM, OM, NDF, ADF, HC and CE as influenced by hydrothermal treatment was as high as 84.4, 85.0, 95.0, 67.6, 97.7 and 73.1% (r^2 values), respectively (all significant at $P < 0.01$). The contents of the above constituents were decreased by 2.13, 1.92, 1.56, 0.32, 1.23 and 0.53% (% in DM), respectively (all significant at $P < 0.01$) as a result of every 1 kp/cm^2 increase in the hydrothermal pressure. The rate of decrease, therefore, was relatively lower for ADF and CE as compared to other constituents.

The *in sacco* disappearance of DM, OM and various CWC was significantly ($P < 0.01$) increased with the elevation of treatment pressure particularly up to 8 kp/cm^2 (Table 3). At 14 kp/cm^2 pressure treatment, the extent of disappearance of these constituents was, however, markedly reduced compared to most of the lower levels of pressure, yet it was significantly higher ($P < 0.05$) compared to untreated straw. The per cent disappearance of these constituents was significantly higher ($P < 0.01$) at 48 h than at 24 h of incubation. At 8 kp/cm^2 pressure treatment the *in sacco* disappearance of DM, OM, HC and CE during a 48-h incubation period was increased from 53.2 to 77.7, 52.4 to 80.3, 49.5 to 82.0 and 49.2 to 79.3%, respectively, and the corresponding figures for per cent increase over the untreated control values were 46, 52, 82 and 61%. Analysis of the data by a log-linear regression model (Table 4) indicated that the disappearance of DM, OM, HC and CE increased significantly ($P < 0.01$) by 0.23, 0.25, 0.39 and 0.33% points as a result of each 10 per cent increase in the hydrothermal pressure and the corresponding increases in the disappearance of these constituents for every 10 per

cent (equivalent to 6 min) addition in the incubation period were at the rate of 2.93, 3.02, 4.18 and 4.84 per cent points ($P < 0.01$).

The data on *in vitro* fermentation by rumen microorganisms indicated that the production of TVFA was significantly ($P < 0.05$) increased on hydrothermally treated compared with untreated rice straw (Table 5). Although there was a gradual increase in the concentration of TVFA in IM with increase in treatment pressure, a significant increase occurred at 10–12 kp/cm^2. At 12 kp/cm^2 pressure treatment the per cent increase in the concentration of TVFA during a 2-, 4-, 6- and 8-h incubation period was 2.85, 4.14, 9.35 and 11.5, respectively, over the untreated controls. Data analysis by linear regression model (Table 5) indicated that the concentration of TVFA significantly increased ($P < 0.01$) at the rate of 0.270 mmole/l rumen fluid for each 1 kp/cm^2 increase in hydrothermal pressure. The concentration of NH_3-N in IM was significantly ($P < 0.05$) decreased while that of TCA-insoluble protein significantly ($P < 0.05$) increased on the hydrothermally treated compared with the untreated straw (Table 6). The effect of hydrothermal treatment was more pronounced up to 12 kp/cm^2 pressure. At this treatment pressure the concentration of NH_3-N during a 2-, 4-, and 6-h incubation period was decreased by 27.0, 37.2 and 28.0%, respectively, while that of TCA-insoluble protein during a 6-, 8- and 12-h incubation period increased by 7.33, 4.37 and 17.8%, respectively, over the untreated values. Estimated parameters of the applied linear regression model (Table 6) indicated that the concentration of NH_3-N was decreased by 0.109 mg/ 100 ml rumen fluid ($P < 0.01$) and TCA-insoluble protein increased by 1.125 mg/ 100 ml rumen fluid ($P < 0.01$) as a result of each addition of 1 kp/cm^2 in the hydrothermal pressure.

Discussion

The substantial decrease in the contents of NDF, HC and CE on hydrothermal treatment of rice straw (Table 1) indicated that this treatment had broken down various chemical bonds between lignin and cell wall polysaccharides and caused further degradations of polymers. The lesser decrease in the content of ADF suggested lesser solubilization of lignin with this treatment. These findings also partly supported the earlier observations where steam treatment given to paddy straw at 9 kg/cm^2 for 30 min (Rangnekar et al., 1982) and at 2 kg/cm^2 for 2 h (Rai and Mudgal, 1986) resulted in a decrease in the contents of DM, OM and various cell wall polysaccharides.

In this investigation, the *in sacco* disappearance included a portion already solubilized by hydrothermal treatment as well as a rumen-degraded residue portion. The data suggested that hydrothermal treatment of rice straw at 8–10 kp/cm^2 pressure significantly increased the *in sacco* disappearance of DM, OM and various CWC (Table 3). The lesser disappearance of DM, OM and CWC at treatment

Table 1

Effect of hydrothermal treatment of rice straw on the content (% in DM) of dry matter, organic matter and cell wall constituents

Treatment pressure (kp/cm^2)	Dry matter	Organic matter	Neutral detergent fibre	Acid detergent fibre	Cellulose	Hemicellulose
0	100	87.3	69.3	45.2	34.3	24.1
1	84.3 (15.7)	73.8 (15.5)	64.5 (6.9)	43.2 (4.4)	34.0 (0.87)	21.2 (12.0)
2	81.3 (18.7)	71.4 (18.2)	65.8 (5.0)	45.0 (0.44)	33.2 (3.2)	20.8 (13.7)
4	80.0 (20.0)	70.0 (19.8)	62.2 (10.2)	43.1 (4.6)	31.5 (8.2)	19.1 (20.7)
6	77.6 (22.4)	67.4 (22.8)	59.3 (14.4)	42.4 (6.2)	30.0 (12.5)	16.9 (29.9)
8	71.8 (28.2)	63.0 (27.8)	54.4 (21.5)	40.2 (11.1)	29.2 (14.9)	14.2 (41.1)
10	67.5 (32.5)	56.5 (35.3)	50.0 (27.8)	40.6 (10.2)	25.7 (25.1)	9.36 (61.2)
12	63.3 (36.7)	55.5 (36.4)	48.9 (29.4)	41.0 (9.3)	27.0 (21.3)	7.9 (67.2)
14	64.3 (35.7)	56.0 (35.8)	48.0 (30.7)	40.8 (9.7)	29.2 (14.9)	7.2 (70.1)
Calculated F-values (Treatments)	90.42*	79.51*	58.77*	12.39*	25.02*	489.06*
C.D. ($P<0.05$) (Treatments)	3.89	3.74	3.33	1.71	1.94	0.91

Values are the mean of two replications. Figures in parentheses represent per cent decrease/solubility over control values. In this and subsequent tables, values marked * and ** are significant at $P < 0.05$ and $P < 0.01$, respectively

Table 2

Estimates of the linear regression model showing the effect of hydrothermal treatment of rice straw on the content (% in DM) of dry matter, organic matter and cell wall constituents

Constituents (Y)	Estimates of linear regression model y = a + bx, n = 18		
	a	b	r^2
Dry matter	90.0636** (1.8014)	–2.1252** (0.2289)	0.8435**
Organic matter	78.8963** (1.5925)	–1.9233** (0.2023)	0.8496**
Neutral detergent fibre	67.8387** (0.7043)	–1.5602** (0.0895)	0.9500**
Acid detergent fibre	44.4164** (0.4355)	–0.3194** (0.0553)	0.6756**
Cellulose	33.7675** (0.6295)	–0.5276** (0.0800)	0.7311**
Hemicellulose	23.3785** (0.3880)	–1.2327** (0.0493)	0.9751**

Abbreviations used in regression model in this and subsequent tables are: a = intercept, b = regression coefficient of treatment pressure, x = hydrothermal treatment pressure (kp/cm^2), r^2 = coefficient of determination, x1 = hydrothermal treatment pressure (kp/cm^2), x_2 = incubation period (h), R^2 = coefficient of determination, b0 = intercept, b1 and b2 = regression coefficient on x1 and x2, respectively, and n = number of observations. Figures in parentheses denote standard error of regression coefficients. ** significant at $P < 0.01$

Table 3

Effect of hydrothermal treatment of rice straw on *in sacco* dry matter, organic matter and cell wall constituents disappearance (%) in the rumen

Treatment pressure (kp/cm^2)	Constituents/period of rumen incubation (h)							
	Dry matter		Organic matter		Cellulose		Hemicellulose	
	24	48	24	48	24	48	24	48
0	40.8	53.2	38.7	52.4	29.5	49.2	26.6	49.5
*1	47.4	54.0	51.7	57.1	36.1	50.2	37.7	66.7
2	52.2	65.1	50.7	65.0	46.8	71.0	48.9	66.0
4	57.0	67.1	57.0	68.3	55.0	75.0	62.5	70.0
6	61.1	68.1	62.3	66.5	63.5	77.5	72.8	74.4
8	70.7	77.7	70.5	80.3	75.0	79.3	77.5	82.0
10	67.0	75.1	67.5	73.2	67.5	76.2	49.6	69.6
12	62.3	89.5	65.2	95.3	62.1	87.4	ND	ND
14	48.2	69.9	47.1	69.9	31.3	64.8	ND	ND
Calculated F-values								
Treatments	321.2**		1813.3**		2968.6**		1482.4**	
Incubations	1138.2**		5948.7**		11040.4**		2930.4**	
Interaction	42.3**		288.1**		267.1**		208.3**	
CD ($P < 0.01$)								
Treatments	1.08		0.49		0.51		0.82	
Incubations	2.29		1.04		1.07		1.48	
Interactions	3.23		1.47		1.52		2.09	

Values mean of two replications. ND: not determined. * In this and subsequent tables: treated for 30 min

Table 4

Estimates of the log-linear regression model showing the effect of hydrothermal treatment of rice straw on *in sacco* dry matter, organic matter and cell wall constituents disappearance (%) in the rumen

Constituents (Y)	Estimates of log linear regression model $Y = b_0 x_1^{b1} x_2^{b2}$, n = 36			
	b_0	b_1	b_2	R^2
Dry matter	21.8667 (1.2446)	0.0225** (0.0043)	0.2933** (0.0618)	0.6001**
Organic matter	21.4070 (1.2532)	0.0252** (0.0045)	0.3018** (0.0637)	0.6213**
Cellulose	10.6112 (1.4645)	0.0333** (0.0076)	0.4835** (0.1077)	0.5448**
Hemicellulose	13.8326 (1.4148)	0.0388** (0.0063)	0.4182** (0.0980)	0.6905**

Table 5

Effect of hydrothermal treatment of rice straw on *in vitro* total volatile fatty acids production (mmole/l rumen fluid) by rumen microorganisms from buffalo calves

Treatment pressure (kp/cm^2)	Incubation time (h)				
	2	4	6	8	12
0	63.2	67.6	72.7	65.5	78.6
*1	63.3	67.5	72.7	65.5	77.4
2	63.3	67.6	74.5	66.0	75.5
4	63.5	67.7	74.6	66.0	76.9
6	63.9	68.0	74.8	66.0	77.0
8	64.2	68.1	75.0	66.6	76.8
10	64.4	69.3	76.7	69.1	76.8
12	65.0	70.4	79.5	72.8	76.6
14	65.0	70.5	79.6	73.0	76.7

Analysis of variance

Sources of variation	df	F-ratio	CD ($P < 0.05$)
Treatment	8	5.22*	1.8319
Incubations	4	134.07*	1.3654

Linear regression equation: $Y = b_0 + b_1x_1 + b_2x_2$, $n = 45$

$= 62.0329^{**} + 0.2697^{**} + 1.0661^{**}$ $R2 = 0.5501^{**}$

(1.3562) (0.1149) (0.1575)

Table 6

Effect of hydrothermal treatment of rice on *in vitro* production of ammonia (mg NH_3–N/100 ml rumen fluid) and biosynthesis of protein (mg protein/100 ml rumen fluid) by rumen microorganisms from buffalo calves

Treatment pressure (kp/cm^2)	Constituent/incubation time (h)							
	Ammonia-N					Protein		
	2	4	6	8	12	6	8	12
0	5.99	7.21	8.22	6.59	5.50	150	160	140
*1	5.92	6.82	7.77	6.26	5.53	150	160	142
2	5.66	6.43	7.39	6.19	5.23	150	162	145
4	5.38	6.27	7.23	6.13	5.18	153	163	148
6	5.01	6.11	7.11	6.05	5.24	156	164	155
8	4.65	5.58	6.58	5.91	5.21	160	164	157
10	4.37	5.16	6.08	5.82	5.27	160	166	160
12	4.37	4.53	5.92	5.89	5.32	161	167	165
14	4.35	4.53	5.92	5.93	5.26	162	167	164

Sources of variation	Analysis of variance			Sources of variation	Analysis of variance		
	df	F-ratio	CD ($P < 0.05$)		df	F-ratio	CD ($P < 0.05$)
Treatments	8	0.29*	0.5193	Treatments	8	7.78*	6.1234
Incubations	4	28.81*	0.3871	Incubations	2	22.36*	3.5353

Linear regression equation:

$Y = b_0 + b_1x_1 + b_2x_2$

$$= \underset{(0.2857)}{6.5224} \overset{**}{-} \underset{(0.0242)}{0.1093}\ x_1 \overset{**}{+} \underset{(0.0329)}{0.0032}\ x_2 \quad (NS)$$

$R^2 = 0.3277$ $\quad n = 45$

Linear regression equation:

$Y = b_0 + b_1x_1 + b_2x_2$

$$= \underset{(3.9347)}{157.2320} \overset{**}{+} \underset{(0.2164)}{1.1250}\ x_1 \overset{**}{-} \underset{(0.4090)}{0.7976}\ x_2 \quad (NS)$$

$R^2 = 0.5623$ $\quad n = 27$

pressures higher than 10 kp/cm^2 may be due to the fact that the insoluble portion of these constituents left after hydrothermal treatment at a pressure beyond 10 kp/cm^2 became more resistant to ruminal microbial degradation. The results of this investigation also supported the earlier work when steam treatment of rice straw (ranging from 7.0 to 42.3 kg/cm^2) at 21 kg/cm^2 increased the *in vitro* digestibility of DM maximally and then decreased it at a higher treatment pressure (Hart et al., 1980), and that at 2 kg/cm^2 for 1 and 2 h significantly increased the *in vitro* digestibility values of DM, CWC, CE while that of ADF remained constant (Rai and Mudgal, 1986).

Increase in the concentration of TVFA in the IM on treated over untreated rice straw (Table 5) may be due to the more expressed solubilization and higher rumen degradation of treated straw (Tables 1 and 3). A higher increase in the concentration of TVFA at early incubation times in the case of treated compared with untreated straw may be the result of quicker fermentation of the treated straw (both soluble and residual portion). The lower concentration of NH_3-N on treated straw may be due to its higher utilization for rumen microbial protein synthesis. An alternative explanation for lower NH_3-N concentration is that hydrothermal treatment decreased the microbial degradability of rice straw protein. This fact is confirmed by the higher content of TCA-insoluble protein in the IM when treated straw was the substrate compared with untreated straw (Table 6).

This study suggests that hydrothermal treatment of rice straw at 14 kp/cm^2 pressure (major effect up to 10 kp/cm^2) significantly solubilized the cell wall constituents and also improved their rumen digestibility. There was a significantly higher production of TVFA by rumen microorganisms and higher TCA-insoluble protein in rumen fluid on treated compared with untreated rice straw.

References

Barnett, A. J. G. and Reid, R. C. (1957): Studies on production of VFA from grasses by rumen liquor in an artificial rumen. I. The VFA production from fresh grasses. J. Agric. Sci. Camb. **48,** 315–321.

Goering, H. K. and Van Soest, P. J. (1970): Forage fibre analysis. Agricultural Handbook No. 379, Washington, D. C., pp. 1–12.

Hart, M. R., Walker, H. G. Jr., Graham, R. P., Hanni, P. J., Brown, A. H. and Kohlar, G. O. (1980): Steam treatment of crop residues for increased ruminant digestibility. I. Effect of process parameters. J. Anim. Sci. **51,** 402–408.

Johnson, R. R. (1966): Techniques and procedures for *in vitro* and *in vivo* rumen studies. J. Anim. Sci. **25,** 855–875.

Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951): Protein measurement with Folin phenol reagent. J. Biol. Chem. **193,** 265–275.

Mehrez, A. Z. and Orskov, E. R. (1977): A study of the artificial fibre bag technique for determining the digestibility of feeds in the rumen. J. Agric. Sci. Camb. **88,** 645–650.

Oji, U. I. and Mowat, D. N. (1978): Nutritive value of steam treated corn stover. Can. J. Anim. Sci. **58,** 177–181.

Oji, U. I. and Mowat, D. N. (1979): Nutritive value of thermoammoniated and steam-treated maize stover. I. Intake, digestibility and nitrogen retention. Anim. Feed Sci. Technol. **4,** 177–186.

Okamoto, M. and Hidenori, A. (1989): Effect of ammonia treatment, steam treatment and ammoniation after steam treatment on chemical composition, voluntary intake and digestibility of rice straw. Nippon Chikusan Gakkaiho **60,** 1117–1121.

Rai, S. N. and Mudgal, V. D. (1986): Effect of alkali and steam pressure treatments on compositional changes and *in vitro* fibre digestibility of rice straw. Indian J. Anim. Nutr. **3,** 95–101.

Rangnekar, D. V., Badve, V. C., Kharat, S. T., Sobale, D. N. and Joshi, A. L. (1982): Effect of high pressure steam treatment on chemical composition and digestibility *in vitro* of roughages. Anim. Feed Sci. Technol. **7,** 61–70.

Sharma, C. P., Kaushal, G. P., Sareen, V. K., Singh, Sudarshan and Bhatia, I. S. (1981): The *in vitro* metabolism of flavonoids by whole rumen contents and its fractions. Zbl. Vet. Med. A **28,** 27–34.

Weatherburn, M. W. (1967): Phenol–hypochlorite reaction for determination of ammonia. Anal. Chem. **39,** 971–974.

COMING EVENTS

THE ROYAL VETERINARY COLLEGE
University of London
presents
a series of Continuing Education Activities

For 1995

Monday 16th – Friday 20th January:
A Five Day Course on Cattle Breeding and Fertility. Venue: Potters Bar

Monday 23rd – Friday 27th January:
A Five Day Course on Cattle Medicine. Venue: Potters Bar

Monday 30th January – Friday 10th February:
A Two Week Course on Sheep Production and Health. Venue: Potters Bar

Monday 27th February – Thursday 9th March:
A Two Week Course on Poultry Diseases. Venue: Compton Laboratory, Berks

Monday 27th February – Friday 3rd March:
A Five Day Course on Diseases of Rats and Mice. Venue: Camden Town

Wednesday 16th, Thursday 17th, Friday 18th March:
A Course on Diseases of Rodents (excl. Rats and Mice) and Amphibians. Venue: Camden Town

Friday 28th April:
A One Day Course on Small Animal Ophthalmology (Lens). Venue: Camden Town

Tuesday 2nd May – Friday 12th May:
A Two Week Course on Pig Diseases. Venue: Potters Bar

Monday 15th May – Friday 19th May:
A Five Day Course on the Microbiology of Foods of Animal Origin. Venue: Camden Town

Monday 22nd May – Thursday 25th May:
A Course on Aspects of Veterinary Public Health, including a two day course on Zoonoses. Venue: Camden Town

Friday 20th October:
A One Day Course on Small Animal Ophthalmology (Uveal Tract). Venue: Camden Town

For further information please contact:

Maggie McEvoy
UVCE, The Royal Veterinary College
Royal College Street, London NW1 0TU
Tel 0171–387 2898 ext 351 Fax 0171–383 0615

PRINTED IN HUNGARY

Akadémiai Kiadó és Nyomda, Budapest

MAGYAR
TUDOMÁNYOS AKADÉMIA
KÖNYVTÁRA

INSTRUCTIONS TO AUTHORS

Manuscripts are accepted on the basis of scientific significance and suitability for publication on the understanding that they have not been published, submitted or accepted for publication elsewhere. Acceptance depends on the opinion of two referees and the decision of the Editorial Board. Papers accepted for publication are subject to editorial revision. Only original papers will be published and a copy of the Publishing Agreement will be sent to the authors of papers accepted for publication. Manuscripts will be processed only after receiving the signed copy of the agreement.

MANUSCRIPT STYLE

Manuscripts must be in English or Hungarian and clearly and concisely written. They should be typed double spaced with wide margins. Two copies of the manuscript should be submitted.

FORM OF MANUSCRIPT

Title. The title should be a clear and concise statement of the contents in not more than 14 words. A short running title of not more than 50 letters should also be supplied. This is followed by the authors' initials (full first name of women) and surname, and the name of the institution where the work was done. The mailing address of the authors must also be indicated here.

Abstract. This should not exceed 200 words and should outline briefly the purpose of the study and detail important findings and the authors' principal conclusions. Redundant phrases, generally known information and repetition should be avoided. Four or five keywords should also be supplied.

Introduction. This part should state briefly the nature and purpose of the work and cite recent important work by others.

Materials and methods. Describe materials, methods, apparatus, experimental procedure and statistical methods in sufficient detail to allow other authors to reproduce the results. This part may have subheadings. The experimental methods and treatments applied shall conform to the most recent guidelines on the humane treatment and care of animals.

Results. The experimental data should be represented clearly and concisely. Avoid repeating information presented in tables and figures.

Discussion should be focussed on the interpretation of experimental findings. Do not repeat literary data presented in the Introduction or information given in Results. References should be cited as follows: e.g. ... as observed by Faith and Trump (1976); or in parentheses: ... were found (Starr et al., 1978; Manson and Starr, 1979).

Acknowledgement of grants and technical help.

References. Cite only essential references. They should be arranged in alphabetical order of the authors' surnames, without serial numbers. The reference list at the end of the paper should contain

- for journals: names and initials of all authors, year of publication (in parentheses), colon, English title of the paper (if the original title is not English, indicate in parentheses, e.g. [in French]), journal title abbreviated according to the style used in Index Veterinarius, volume number, issue number in parentheses, first and last pages;
- for books: names and initials of authors/editors, year (in parentheses), title, publisher, place of publication, page number.

Tables. They should be typed on separate sheets and have a concise heading each. Tables are to be numbered sequentially by Roman numbers.

Figures. Good-quality glossy prints a half-tone illustrations and clear line drawings in Indian ink are accepted. The number of the figure, the author's name and the top of the figure should be indicated lightly in soft pencil on the back. The figures are to be numbered sequentially by Arabic numbers. Captions should be provided on a separate sheet. All figures should be referred to in the text and their approximate place indicated on the margin.

Abbreviations and symbols. Quantities should be expressed in SI units. All abbreviations should be spelled out when first used in the text. Please identify unusual symbols in the margin.

Proofs and reprints. One set of proofs will be provided, which is requested to be returned within 48 hours of receipt to the Editor. Alterations in the text and especially in the illustrations are expensive and should be avoided. Fifty reprints of each paper are supplied free of charge. Additional reprints can be ordered at cost price at the time the page proof is returned.

307209

Acta Veterinaria Hungarica

20.

VOLUME 43, NUMBERS 2—3, 1995

EDITOR–IN–CHIEF
J. MÉSZÁROS

ASSOCIATE EDITOR
A. SZÉKELY

EDITORIAL BOARD
A. BARTHA, J. BOKORI, T. KASSAI, F. KUTAS,
G. PETHES, P. RUDAS, L. SOLTI, S. TUBOLY,
J. VARGA, F. VETÉSI. K. VÖRÖS



Akadémiai Kiadó, Budapest

ACTA VET. HUNG. 43 (2—3) 193—367 (1995) HU ISSN 0236–6290

ACTA VETERINARIA

A QUARTERLY OF THE HUNGARIAN ACADEMY OF SCIENCES

Acta Veterinaria publishes reviews and original papers on veterinary science in English.

Acta Veterinaria is published in yearly volumes of four issues by

AKADÉMIAI KIADÓ

Publishing House of the Hungarian Academy of Sciences

H–1117 Budapest, Prielle Kornélia u. 19—35.

Manuscripts and editorial correspondence should be addressed to

Acta Veterinaria

Veterinary Medical Research Institute of the
Hungarian Academy of Sciences

H–1581 Budapest, P.O. Box 18

Subscription information

Orders should be addressed to

AKADÉMIAI KIADÓ

H–1519 Budapest, P.O. Box 245

Subscription price for Volume 43 (1995) in 4 issues US$ 96, including normal postage, airmail delivery US$ 20.00.

Acta Veterinaria is indexed in Current Contents

Acta Veterinaria Hungarica is abstracted/indexed in AGRICOLA, Biological Abstracts, Bibliography of Agriculture, Chemical Abstracts, Current Awareness in Biological Sciences, Current Contents–Agriculture, Biology and Environmental Sciences, Dairy Science Abstracts, Excerpta Medica, Focus On: Veterinary Science and Medicine, Helminthological Abstracts, Index Medicus, Index Veterinarius, International Bibliography of Periodical Literature, La Presse Médicale, Nutrition Abstracts and Reviews, Review of Plant Pathology, Soils and Fertilizers, Veterinary Bulletin.

"This periodical is included in the document delivery program THE GENUINE ARTICLE of the Institute of Scientific Information, Philadelphia. The articles published in the periodical are available through *The Genuine Article* at the Institute for Scientific Information, 3501 Market Street, Philadelphia PA 19104."

© Akadémiai Kiadó, Budapest 1995

CONTENTS

Animal nutrition

Complex study of the physiological role of cadmium III. Cadmium loading trials on broiler chickens. *Bokori, J., Fekete, S., Kádár, I., Koncz, J., Vetési, F.* and *Albert, M.* 195

Effect of avoparcin on rumen fermentation and duodenal nutrient flow in sheep. *Fébel, Hedvig, Romváry, A., Zsolnai-Harczi, Ildikó* and *Huszár, Szilvia* 229

Animal reproduction

Seasonal changes in sperm parameters of British milk rams. *Sarlós, P.* and *Molnár, A.* 247

Mycotoxin research

Occurrence of *Fusarium* species and zearalenone in dairy cattle feeds in Vojvodina. *Škrinjar, M., Stubblefield, R. D., Stojanović, E.* and *Dimić, G.* 259

Parasitology

Redescription of *Goussia neglecta* n. comb. (Nöller, 1920) (Apicomplexa; Coccidia) and notes on its occurrence in the gut of tadpoles. *Molnár, K.* 269

Effect of exposure to malachite green solution on common carp fry with *Dactylogyrus vastator* (Monogenea) infection. *Molnár, K.* 277

Prevalence of botfly larvae and lice in studs of North Caucasus (Stawropol County, Russia). *Egri, B., Sárközy, P.* and *Bánhidy, Gy.* 287

Pathophysiology

Betamethasone and adult rat lung surfactant lipids. *Stettner, S.* and *Ledwożyw, A.* 291

The effect of betamethasone on phosphatidylcholine species composition in fetal rat lungs. *Stettner, S.* and *Ledwożyw, A.* 297

Prenatal starvation, betamethasone and lung development in newborn rats. *Stettner, S.* and *Ledwożyw, A.* 303

Protective effect of Verapamil on regional myocardial ischaemic injury in dog. *Kobusiewicz, W., Ogonowska-Kobusiewicz, Maria, Michalak, J., Żywicki, W.* and *Ledwożyw, A.* 311

MAGYAR
TUDOMÁNYOS AKADÉMIA
KÖNYVTÁRA

Toxicology

Toxicological studies on potentiated ionophores in chickens I. Tolerance study. *Lehel, J., Laczay, P., Móra, Zsuzsa* and *Semjén, G.* 321
Toxicological studies on potentiated ionophores in chickens II. Compatibility study. *Lehel, J., Laczay, P., Móra, Zsuzsa* and *Semjén, G.* 335
Toxicological studies on potentiated ionophores in chickens III. Electrotoxicological investigations. *Lehel, J.* and *Laczay, P.* 347
Effects of repeated oral doses of Dikamin D (2,4-D-amine Na) on rats. *Várnagy, L., Somlyay, I., Budai, P.* and *Varga, T.* 355
Experimental furazolidone toxicosis in broiler chicks: Effect of dosage, duration and age upon clinical signs and some blood parameters. *Zaman, Q., Khan, M. Z., Islam, N.* and *Muhammad, G.* 359

Acta Veterinaria Hungarica 43 (2–3), pp. 195–228 (1995)

COMPLEX STUDY OF THE PHYSIOLOGICAL ROLE OF CADMIUM III. CADMIUM LOADING TRIALS ON BROILER CHICKENS*

J. BOKORI[1], S. FEKETE[1], I. KÁDÁR[2], J. KONCZ[2], F. VETÉSI[3] and M. ALBERT[3]

[1]Department of Animal Nutrition and [3]Department of Pathology, University of Veterinary Science, H–1400 Budapest, P.O. Box 2, Hungary; [2]Agrochemical and Plant Nutrition Department, Soil Research Institute of the Hungarian Academy of Sciences, Budapest, Hungary

(Received January 10, 1995)

Cadmium (Cd) loading trials were conducted on a total of 110 (3×10 and 4×20) broiler chickens prereared for 21 days. The control chickens received no cadmium, while chickens in the six treatment groups were given different doses of Cd as an aqueous solution of $CdSO_4$ administered either into the crop or mixed in the feed. The chickens were kept in a climatized animal house and treated usually for 3–5 weeks (maximum 68 days), with the exception of group Cd-75 chickens which were treated up to 239 days of age. The chickens' health status, body mass and feed consumption were monitored throughout the trial. On days 14–20 and on day 42 of the trial 2 chickens per group, then at the end of trial a total of 25 chickens were killed in anaesthesia. These birds, together with chickens that died or were killed during the trial, were subjected to detailed gross pathological examination. From 11 organs (kidney, liver, spleen, testicle, brain, myocardium, skeletal muscle, lungs, digestive tract, pancreas, tubular bones) of these chickens samples were taken for assay for a total of 16 elements, as well as for light and electron microscopic examination. With the exception of groups Cd-30 and Cd-600, no abnormal clinical signs were observed in the first two weeks of the trial. Chickens of group Cd-30 died before day 8–12 of the trial among signs of complete anorexia, rapid emaciation, huddling and diarrhoea, while chickens of group Cd-600 died before day 28, showing similar clinical signs. The body mass of chickens fed a Cd-supplemented diet either remained constant or decreased substantially, in a degree proportional to the Cd load. The only exception was group Cd-2.5, in which the average body mass of birds at the end of week 8 slightly exceeded that of the controls. Four out of the 10 cockerel chicks fed a diet containing 75 ppm Cd up to 239 days of age died of intercurrent diseases; the remaining six grew well and reached a body mass of 3.8–4.3 kg. Feed conversion efficiency was satisfactory in the control group and in group Cd-2.5 (2.1 and 2.4 kg, respectively) and could not be evaluated in a realistic manner in the other groups. At necropsy, the cockerel chicks of groups Cd-30 and Cd-600 showed severe emaciation, liver and kidney degeneration, myocardial hypertrophy and cardiac dilatation. In chickens of groups Cd-75 and Cd-

*This study was supported by the National Research Fund (OTKA), project no. 1270

0236–6290/95/$ 4.00 © 1995 *Akadémiai Kiadó, Budapest*

300, a body mass reduction, hepatic and renal degeneration, pancreatic, lienal and testicular atrophy and cardiac hypertrophy were observed. In chickens exposed to prolonged Cd load, the glandular stomach and the gizzard were dilated and filled with a mucinous substance; the wall of the gizzard was thinned and its keratinoid layer thickened. In chickens exposed to a daily Cd load of 2.5 mg, which developed well, the substantial hypertrophy and oedematous infiltration of the testicles was a conspicuous finding. In cocks fed Cd for 239 days the above-mentioned changes of the parenchymal organs were accompanied by the presence of bones which were easy to cut through. Microscopic findings included more or less severe tubulonephrosis in the kidneys of almost all chickens exposed to Cd load. In individuals treated with higher doses of Cd for a long period fibrosis was also present. In the livers there was necrosis and biliary duct proliferation; in the spleens an atrophy of the Malpighian corpuscles, in the testicles a damage of the germinal epithelium and, upon higher Cd load, severe atrophy. In the brain there were areas with gliosis and malacia, the skeletal muscles showed oedema and muscle fibre atrophy, and in the myocardium a reactive necrosis could be seen. In the dilated parabronchi of the lungs a mucinous substance had accumulated, the walls of the air and blood capillaries had thinned down and ruptured. In the glandular stomach the glands of the tunica propria, while in the gizzard the mucosal glands were atrophied. The epithelial cells of the glandular chambers of the pancreas were also atrophic. In chickens exposed to a lower Cd load the bones showed a calcification disturbance of the degenerated epiphyseal cartilage, while in those subjected to a higher Cd load osteoporosis, osteoclastic osteolysis and bone marrow aplasia developed. Electron microscopic examination revealed the welling of mitochondria and endoplasmic reticula in the hepatocytes. The glandular epithelial cells of the pancreas became depleted in granules, the cisternae of the RER were dilated, and the cytoplasm showed partial necrosis and the appearance of autophagous vacuoles. In the myocardium, dilatation of the sarcoplasmic reticula, oedema, and the formation of giant mitochondria were observed. The tubular epithelial cells of the kidneys contained hyaline droplets and showed mitochondrial degeneration, the nerve cells of the brain became depleted in glycogen and showed a dilatation of the RER cisternae. Depending on the degree of Cd load, the concentration of Cd increased in all organs. In the Cd-300 group, the Cd concentration of the liver (667.80 mg/kg) was approx. 4000 times higher than in the control group (0.15 mg/kg). The Cd concentration of the kidney also rose by three orders of magnitude, and reached a maximum of 333.0 mg/kg. Cd accumulation was very substantial in the spleen (20.57 mg/kg), testicle (17.50 mg/kg) and pancreas (83.86 mg/kg). The Cd content of the bones showed a less marked increase, with a maximum of 4.66 mg/kg. As a result of the Cd load, the Zn content of the bones appreciably decreased. Cd loading exerted a positive or negative effect on the metabolism of several microelements (Fe, Zn, Cu, Mn, Mo, B, Sr). Upon Cd loading, the content of P increased in all organs except the spleen, testicles and the lungs. The same was found for the Ca content of all organs but the bones, the Ca content of which slightly decreased as a result of higher Cd load.

Key words: Broiler chicken, cadmium, load, accumulation, poisoning, gross pathology, histopathology

Due to its growing use, cadmium (Cd), an element ubiquitous in nature, has become an increasingly important source of environmental pollution: it gets into the organism of plants and animals and accumulates in their organs in growing quantities. The Cd content of feed plants depends on the quality of arable soil and the species of plant. Different parts of the plant accumulate dissimilar quantities of Cd: the decreasing order is root, stalk, leaf, and seed-crop. Cd may enter the animal organism from three sources (feed, drinking water, air) by two different routes (per os and by inhalation).

The degree of Cd absorption from the intestine depends on the Cd compound, the composition and physical condition (pH) of the intestinal content, the ratio of organic substances contained by it, the presence of other metals such as Fe (Fox, 1974) and Ca (Washko and Cousins, 1976); however, it is also influenced by the species, sex (Anke et al., 1982) and age (Bunker et al., 1984) of the animal. Only about 50% of the Cd entering the organism via the respiratory route will reach the alveoli and become absorbed. Cd exposure of this type (by inhalation) has hardly any importance in animals, first of all in birds.

Due to its long excretion period and difficult measurability, it is not easy to study the metabolism of Cd. In spite of this fact, its absorption and accumulation in different organs have been studied by several authors (Freeland and Cousins, 1973; Pritzl et al., 1974; Sumino et al., 1975; Learch et al., 1979; Sharma et al., 1979; Sas and Tu, 1989).

Although several researchers (Flick et al., 1971; Fassett, 1972; Anke et al., 1978) have referred to the biological role of Cd, its essentiality has not been proved to this very day. Recently it has been assigned to those elements whose physiological, biochemical and biophysical effects have partially been established (Frieden, 1985; Pais, 1989). According to most experts (Black et al., 1979; Hapke, 1992), while Cd deficiency does not occur under practical conditions, the uptake of Cd usually leads to major toxic consequences (Régius-Môcsényi et al., 1985; Hapke, 1992). Birds have been shown to be less sensitive to Cd exposure (Hapke, 1975).

In poultry, a single oral uptake of a large dose of Cd leads to mortality due to severe acute damage of the parenchymal organs and preceded by nervous symptoms.

Prolonged intake of Cd in smaller quantities causes chronic lesions in the digestive tract, testicles, liver, kidney, myocardium and bones (Bokori and Fekete, 1995).

Observations on the accumulation of Cd within the organism, its interaction with other minerals, its toxicity, absorption, metabolism, physiological effects, ac-

cumulation in the organism, excretion, and the results of feeding trials conducted in some animal species have been reported in a separate paper (Bokori and Fekete, 1995). The results of some Cd exposure experiments performed in poultry by other authors are summarized below.

Pritzl et al. (1974) conducted 20-day Cd loading experiments on 196 two-week-old Leghorn cockerels. The 100 chickens included in the first experiment were assigned to five groups fed, *ad libitum*, a diet containing different levels of Cd (0, 400, 600, 800 and 1000 ppm) added in the form of $CdCO_3$. In the second experiment, 96 chickens were assigned to four groups. The feed of two groups did not contain Cd (control groups), while that of the two other groups was supplemented with 700 ppm Cd. The main findings obtained by clinical, gross pathological, histopathological examinations and chemical analysis were as follow: (*i*) In chickens fed 700 ppm Cd, the relative weight of the gastrointestinal tract, kidney and gizzard lining increased. (*ii*) The colour and mineral content of several organs changed, and their Cd and Zn content markedly increased. The atrophic liver showed focal degeneration and in the hepatocytes karyolysis, karyorrhexis and karyopycnosis were observed. (*iii*) The body mass gain of the 14–34 days old chickens decreased and their feed conversion rate deteriorated in proportion to the increasing Cd load. (*iv*) In commercial diets, a Cd content of more than 400 ppm was expressly toxic. Chickens fed a diet containing 800 and 1000 ppm Cd died before the end of the feeding trial. (*v*) From these results, Pritzl et al. (1974) concluded that in 2-week-old chickens the LD_{50} of Cd fed in the diet over a period of several days was 565 ppm, and was influenced also by the higher Zn content of the diet.

Sharma et al. (1979) fed a diet containing 0.3, 1.9 or 13.1 ppm Cd to Leghorn laying hens for 6 months. After such Cd load no Cd accumulation was observed either in the bones or in the egg. In the group exposed to the highest Cd dose, in the 6th month of the trial an accumulation of Cd was demonstrated in the muscles (the Cd content rose from 0.32 mg/kg to 1.9–13.0 mg/kg). The highest Cd concentrations were measured in the liver and kidney (11.0 and 42.0 mg/kg fresh material). The measured Cd concentrations closely depended on the dose and the duration of exposure.

Leach et al. (1979) studied dietary Cd exposure of broiler chickens and 6- to 8-month-old laying hens and monitored the accumulation of Cd in the birds' organs. The chickens were fed $CdSO_4$ supplemented diets of different Cd concentration (0.3, 12.0 and 48 μg/g) *ad libitum*, for 6–12 and 48 weeks. Main findings: (*i*) The feed containing 48 μg/g Cd was considered to be slightly toxic. (*ii*) All three levels of Cd load increased the Cd concentration of the kidney in broilers (from 0.39 μg/g to max. 239 μg/g fat-free dry matter), while only the two higher doses of dietary Cd caused a marked rise in Cd concentration of the liver and muscles (from 0.23 to 87.19 μg/g in the liver and from 0.07 to 0.75 μg/g in the

muscles). (*iii*) The Cd level of the eggs was found to be very low, and a measurable increase in Cd concentration (0.22 μg/g fat-free d.m.) occurred only in the thin-shelled eggs of laying hens fed 48 μg/g Cd.

In experiments on broiler chickens, Ueberschär et al. (1982) studied the ability of Se to decrease Cd toxicity. The diet of one group was supplemented neither with Cd nor with Se (control group), a second group received a diet supplemented with 30 ppm Cd, a third group was fed a diet containing 30 ppm Cd and 10 ppm Se, while a fourth one was given a diet supplemented with 30 ppm Cd and 20 ppm Se. The concentration of Cd was measured in eight organs of the chickens in the 3rd and 6th week of the Cd load. The values indicated are expressed to the wet mass of the organs. Principal findings: (*i*) in the control group, the highest Cd concentration was measured in the feathers (0.52 ppm) and bones (0.06 ppm) in the 6th week of the trial. (*ii*) In chickens fed a diet containing 30 ppm Cd, the concentration of Cd markedly increased in all organs as a function of time: the highest Cd concentration was measured in the kidney (21 ppm) and liver (9.9 ppm) after 6-week Cd exposure. (*iii*) The simultaneous administration of Se consistently reduced the Cd concentration of the organs examined: e.g. in chickens fed 30 ppm Cd and 20 ppm Se the Cd content of the organs was 50–70% lower than in those fed 30 ppm Cd alone.

Bruckner (1988) studied the effect of different feed constituents including sodium phytate and Se on the Cd metabolism of broilers. The presence of these substances in the feed was found to decrease Cd accumulation both in the liver and in the kidney.

Stolley (1989) conducted three trials on a total of 274 day-old Lohman cockerel chicks to follow up the effects exerted by two vitamins (E and B_6) and some microelements (Cu, Se, Mn and Zn) on the accumulation of Cd in the kidney. These feed constituents were administered at varying concentrations and partly in combination. Cd was added to the diet in the form of $CdCl_2$ to give a concentration of 3 ppm. Zn, Cu and Mn were added as acetates while Se as sodium selenite. During the 21-day feeding trial the chickens' general health status, body mass, and feed conversion efficiency were monitored. Crystalline vitamin E and pyridoxine manufactured by Merck were homogeneously mixed in the soybean- and maize-based diet of the different groups. Main findings: (*i*) Vitamin E and pyridoxine fed at different concentrations had no influence on Cd retention. (*ii*) Se administered on one occasion in a dose of 5 ppm decreased Cd retention in the kidney. (*iii*) Changes in dietary Mn concentration had no spectacular effects on kidney Cd content. (*iv*) Elevation of dietary Cu content from 12 to 240 ppm led to significant Cd accumulation in the kidney. This was slightly decreased by the administration of Zn. (*v*) The Cd concentration measured in the kidney was between 616 and 2718 mg/kg dry matter.

As the heavy metals contained in the diet of laying hens are usually excreted also in the eggs laid (Stadelman and Prati, 1989), Holcman and Smodis (1973) studied the appearance of four microelements (As, Cd, Hg and Pb) in the eggs of laying hens kept outdoors and in closed system. By examining lyophilized samples, they found that As, Cd and Hg accumulated in the egg-white while Pb in the egg-yolk. The eggs laid by hens kept outdoors, in an area exposed to Cd pollution contained higher amounts of the four metals than those laid by hens kept indoors and fed commercial diets.

The present paper summarizes the results of experiments conducted to study the biological effects of Cd, its accumulation in the organism, its pathological effects and influence exerted on the metabolism of other elements, and compares them with findings reported in the literature by other authors.

Materials and methods

A total of 3 × 10 and 4 × 20 ROSS meat-type hybrid cockerel chicks (total n = 110) were used in the Cd loading trial. The chicks were selected from among 200 cockerels prereared for 21 days, and were assigned to 7 groups of roughly identical body mass.

The cockerels were labeled with wing numbers and housed, in groups of 10 or 20, on the tiers of three-tier meat-type chicken rearing batteries so as to eliminate any temperature, humidity or illumination differences possibly arising in the climatized chicken house.

In the 21-day prerearing period the chickens received a semi-intensive poultry starter diet (Table 1). From the end of prerearing up to the end of the feeding trial, they were fed a poultry grower diet of a composition and content shown in Table 2. The diet fed to the control birds (Cd-0), which did not contain Cd in detectable quantities, was not supplemented with Cd. Of the six experimental groups, three were treated with different doses of Cd in the form of aqueous $CdSO_4$ solution administered into the crop, while the other three groups were fed a diet into which crystalline $CdSO_4$ had been mixed homogeneously in different doses (Table 3). Diet and drinking water were available to the chickens *ad libitum*.

Treatment with the aqueous solution of Cd usually lasted 3 weeks (max. 68 days), while the Cd-supplemented diet was fed up to 89 days of age. Ten chickens of the Cd-75 group continued to receive Cd for an additional 150 days (total duration of treatment: 239 days) in order to produce chronic Cd poisoning.

The clinical status and feed consumption of the chickens were checked daily throughout the experiment. Their body mass was measured and recorded weekly up to 8 weeks of age (Table 4). Later on, when mass mortality occurred and big inter-group differences emerged in the birds' appetite, this practice was abandoned.

MAGYAR
TUDOMÁNYOS AKADÉMIA
KÖNYVTÁRA

Table 1

Composition and declared content of poultry starter diet

Composition		kg
Maize		40.0
Wheat		30.0
Extracted soybean, 48%		17.5
Fish meal, 70%		5.5
Energomix-50		2.0
Poultry complete premix		5.0
Total:		100.0
Content		
ME	MJ/kg	11.80
Crude protein	%	19.75
Digestible crude protein	%	17.45
Crude fat	%	4.26
Crude fibre	%	2.19
NaCl (added)	%	0.12
Ca	%	1.00
P (available)	%	0.43
Methionine + cystine	%	0.84
Lysine	%	1.08
Linolic acid	%	1.22
Vitamin A	IU/kg	10,125.00
Vitamin D_3	IU/kg	3,025.00
Vitamin E	mg/kg	15.85
Elancoban	mg/kg	100.00

On day 14–20 and day 42 of treatment, 2 birds were killed by bleeding in each group. These chickens, together with the ones that had died of different causes or had been culled, were subjected to detailed gross pathological and microbiological examination. At the end of the feeding trial, when the chickens were 8 weeks old, 2–6 chickens per group (a total of 25 birds) were exsanguinated in anaesthesia, necropsied, and samples were taken from 11 of their organs (kidney, liver, spleen, testicle, brain, myocardium, skeletal muscle, lungs, digestive tract, pancreas, and tubular bones) for analysis for 16 mineral elements (P, Ca, Mg, Na, K, S, Cu, Zn, Fe, Mn, Mo, Cd, Se, B, Sr, and Co) and for light microscopic examination. In addition, from the organs of 2 birds per group samples were taken for transmission electron microscopy.

Table 2

Composition and declared content of poultry grower diet*

Composition		kg
Maize		38.0
Wheat		32.0
Extracted soybean, 48%		17.5
Fish meal, 70%		3.5
Extracted sunflower meal		3.0
Energomix-50		1.0
Poultry complete premix		5.0
Total:		100.0
Content		
ME	MJ/kg	12.46
Crude protein	%	19.00
Digestible crude protein	%	16.58
Crude fat	%	3.57
Crude fibre	%	2.38
NaCl (added)	%	0.18
Ca	%	0.54
P (available)**	%	0.62
Methionine + cystine	%	0.72
Lysine	%	1.01
Vitamin A	IU/kg	10,000.00
Vitamin D_3	IU/kg	2,000.00
Vitamin E	mg/kg	11.00
Elancoban (monensin)	mg/kg	100.00

*The diet of three groups (Cd-75; Cd-300; Cd-600) was supplemented with in the form of $CdSO_4$; **P was supplied as MCP

On day 28 after the start of Cd load, 2 cockerels per group were killed by bleeding and their pancreas and small intestinal content were collected. The pancreas samples were homogenized, then centrifuged. The intestinal content was centrifuged after dilution. The obtained supernatants were used for determination of hydrolase activity. Enzyme activity was expressed for 1 mg protein. The hydrolase activity of the samples was determined by standard procedures.

For light microscopy, organ samples were fixed in 8% neutral formaldehyde solution, then dehydrated and embedded in paraffin. Before embedding, bone samples taken from the femur and from the proximal end of the tibiotarsal bone

were decalcified in Ca-EDTA (pH 7.4). The sections were stained with haemalum-eosin and Endes's trichrome staining.

Table 3

Design of the experiment

Groups	Number of chickens	Treatment	
	(n)	Cd (mg/kg body mass)	Cd (mg/kg of feed)
Cd-0 (control)	20	0	0
Cd-2.5	10	2.5	0
Cd-10	10	10.5	0
Cd-30	10	30.0	0
Cd-75	20	0	75.0
Cd-300	20	0	300.0
Cd-600	20	0	600.0

The samples fixed in 5% buffered glutaraldehyde were processed for electron microscopic examination, the feed samples were assayed for content and Cd concentration, and the organs were assayed for mineral element content as described in an earlier paper (Bokori et al., 1993). The mineral element content of the different organs, calculated on a dry matter basis, was expressed in mg/kg units. Organs from individual birds and in some cases pooled samples from several chickens were used for measuring the concentration of mineral elements. The results of analyses were compiled in tables by group and by organ. For six elements, the average concentrations measured in 10 organs of the birds were also summarized in tables.

Results

The results of the weekly weighings and the effect of increasing Cd load on the fate of chickens are presented in Table 4. The cumulative feed utilization data, the average and extreme values of 16 mineral elements measured in 10 organs of chickens in each treatment group, and the standard deviations and CV% of the results were summarized in 11 tables not included in the present paper. Some data included in these tables were also taken into consideration in the evaluation of results. The accumulation of six minerals (P, Ca, S, Cd, Fe, and Mn) in 10 organs of the chickens exposed to a Cd load of different severity, together with their average concentrations per 1 kg dry matter are presented in Table 5. The concentrations of 8 other elements (Na, K, Mg, Zn, Cu, Mo, Sr and Co) measured in 4 organs (liver, kidney, testicle and bone) of several chickens per group are shown in

Table 6, while the mineral concentrations determined in the blood of some birds are summarized in Table 7.

Table 4

Age, number and average body mass (g) per group of the chickens

Group		Week 3	Week 4	Week 5	Week 6	Week 7	Week 8
Cd-0	n	20	19	19	19	16	16
(control)	x	458.00	550.53	1000.80	1469.47	2074.38	2585.63
	s	79.78	88.41	135.70	218.52	254.82	284.72
	Cv %	17.42	16.06	12.85	14.87	12.28	11.01
	sx	17.84	20.28	31.13	50.13	63.70	71.18
Cd-2.5	n	10	9	7	7	7	7
	x	534.00	631.11	1161.11	1654.30	2185.71	2626.67
	s	17.76	37.90	83.13	13.30	141.86	178.62
	Cv %	33.27	60.05	71.60	80.00	64.91	68.00
	sx	5.62	12.63	27.71	50.26	53.62	72.94
Cd-10	n	10	10	9	7	7	7
	x	529.00	534.00	330.00	229.51	171.00	182.71
	s	27.67	49.26	97.34	118.72	168.82	259.08
	Cv %	52.30	92.25	97.34	91.63	98.73	141.80
	sx	8.75	15.58	32.45	44.87	63.80	97.91
Cd-30	n	10	10	0	0	0	0
	x	535.00	396.00				
	s	20.68	28.75				
	Cv %	38.66	72.61				
	sx	6.54	9.09				
Cd-75	n	20	20	20	20	18	17
	x	458.00	483.50	797.00	1030.30	1306.67	1596.47
	s	80.83	88.63	146.86	191.70	267.25	273.43
	Cv %	17.65	18.33	18.43	18.56	20.45	17.13
	sx	18.07	19.82	32.84	42.84	62.99	66.32
Cd-300	n	20	20	20	20	17	16
	x	458.00	446.00	475.50	475.50	495.88	475.63
	s	80.83	74.30	87.93	75.71	85.08	80.41
	Cv %	17.64	16.66	18.49	15.92	17.16	16.91
	sx	18.07	16.61	19.66	16.93	20.63	20.10
Cd-600	n	20	20	20	20	0	0
	x	458.00	395.50	367.00	353.00		
	s	80.63	68.79	66.89	76.03		
	Cv %	17.60	17.39	18.23	21.54		
	sx	18.03	15.38	14.96	17.00		

Several of the changes found at necropsy and by light as well as electron microscopy are shown in the 4 macro- and 12 microphotographs.

The microbiological examinations consistently yielded negative results.

Table 5

Concentrations of P, Ca, S, Cd, Fe and Mn in 10 organs of broiler chickens treated with cadmium in different doses and in different ways

Groups	Organ	Element (mg/kg dry matter)					
		P	Ca	S	Cd	Fe	Mn
Cd-0	Liver	11,166.4	116.8	7,334.2	0.15*	520.18	14.46
	Kidney	11,256.6	417.4	6,669.0	0.11*	257.80	7.54
	Skeletal muscle	8,266.6	195.2	6,362.0	0.00	23.42	0.47
	Myocardium	8,338.6	257.0	9,489.8	0.00	121.60	1.95
	Brain	12,724.6	475.8	5,421.2	0.00	60.58	1.70
	Spleen	13,443.0	259.4	9,031.0	0.00	359.40	1.73
	Testicle	13,197.0	607.0	4,261.4	0.00	94.00	8.30
	Lungs	9,209.6	267.2	8,482.0	0.00	534.50	1.38
	Pancreas	14,171.4	490.0	6,443.0	0.06*	35.56	6.27
	Bone	56,434.0	116,604.0	726.2	0.10*	117.80	2.20
Cd-2.5	Liver	11,061.5	258.0	7,183.5	48.23	191.88	7.43
	Kidney	10,014.7	370.7	7,136.2	190.13	154.25	6.97
	Skeletal muscle	7,963.7	209.2	7,839.2	0.37	21.75	0.46
	Myocardium	8,371.7	280.0	9,298.2	0.28	125.00	1.33
	Brain	13,060.7	591.0	5,467.5	0.45	63.47	1.91
	Spleen	13,747.7	311.0	8,585.2	3.23	317.75	1.61
	Testicle	13,574.5	855.7	8,811.5	0.99	80.40	6.42
	Lungs	9,379.5	664.5	8,567.2	1.40	512.85	2.13
	Pancreas	12,599.7	517.0	5,902.7	7.06	28.90	4.72
	Bone	58,429.0	121,532.0	776.0	0.60	123.55	1.65
Cd-10	Liver	10,002.5	204.7	6,565.7	142.25	186.75	8.67
	Kidney	10,679.0	429.7	7,870.5	315.75	151.75	8.14
	Skeletal muscle	8,563.7	191.7	7,317.5	0.95	23.95	0.47
	Myocardium	8,397.7	302.5	8,759.7	2.35	111.67	1.91
	Brain	13,496.6	310.7	5,766.5	0.31	67.65	1.79
	Spleen	14,002.2	384.0	8,369.0	9.54	305.50	1.90
	Testicle	11,986.2	2,076.0	9,029.0	4.21	136.28	6.02
	Lungs	9,287.2	570.0	8,188.5	3.66	491.20	2.49
	Pancreas	14,464.7	512.7	5,395.5	20.70	36.55	3.82
	Bone	60,217.0	122,448.0	758.0	1.25	120.03	1.51

*Average of multiple determinations

Table 5 continued

Concentrations of P, Ca, S, Cd, Fe and Mn in 10 organs of broiler chickens treated with cadmium in different doses and in different ways

Groups	Organ	Element (mg/kg dry matter)					
		P	Ca	S	Cd	Fe	Mn
Cd-75	Liver	11,192.2	728.5	7,615.7	172.15	125.85	7.61
	Kidney	11,183.5	444.7	7,616.2	284.00	99.22	7.81
	Skeletal muscle	8,967.7	211.0	7,932.2	1.42	24.18	0.62
	Myocardium	8,198.5	307.5	9,432.7	2.71	99.85	1.59
	Brain	13,361.0	443.7	5,576.0	0.55	61.02	0.26
	Spleen	14,251.0	513.0	8,913.7	13.82	260.00	2.02
	Testicle	12,110.0	1,189.7	10,943.2	11.64	83.35	3.88
	Lungs	9,545.0	643.0	9,356.7	4.18	505.80	2.00
	Pancreas	14,765.7	735.2	6,307.2	20.99	31.35	4.54
	Bone	62,201.0	123,632.0	8,370.2	1.79	113.28	1.36
Cd-300	Liver	11,319.8	715.4	8,592.4	667.80	240.60	7.97
	Kidney	11,306.2	555.4	9,240.6	246.20	94.58	6.69
	Skeletal muscle	9,502.0	264.6	8,515.0	6.94	40.06	0.94
	Myocardium	8,411.8	328.2	9,874.8	9.82	132.00	1.89
	Brain	12,285.0	373.8	5,706.0	1.32	75.50	0.72
	Spleen	8,294.0	258.8	7,988.0	25.50	287.60	1.52
	Testicle	–	–	–	–	–	–
	Lungs	10,848.8	1,025.8	8,140.0	34.48	432.22	4.22
	Pancreas	11,594.6	537.4	8,166.2	83.86	28.92	4.36
	Bone	51,221.0	104,457.0	767.8	4.31	120.76	1.51
Cd-600	Liver	12,070.7	744.3	8,400.0	666.67	379.33	9.87
	Kidney	13,940.7	631.0	8,631.7	333.00	106.47	6.84
	Skeletal muscle	8,706.0	378.3	8,915.0	12.67	64.50	0.98
	Myocardium	8,860.0	486.7	9,989.3	11.25	155.33	1.75
	Brain	14,002.0	833.0	5,450.7	1.33	88.23	2.18
	Spleen	12,036.7	3,295.3	7,835.7	29.57	465.33	1.91
	Testicle	10,936.3	6,825.7	8,412.3	17.50	324.33	12.50
	Lungs	7,503.0	824.7	7,443.3	17.92	606.43	2.72
	Pancreas	16,512.0	681.0	9,608.3	61.67	30.40	4.01
	Bone	65,772.0	125,507.0	874.3	4.66	131.33	1.50

Table 6

Concentrations of Na, K, Mg, Zn, Cu, Mo, Sr and Co in four organs of broiler chickens treated with cadmium in different doses and in different ways

Groups	Organ	Element (mg/kg dry matter)							
		Na	K	Mg	Zn	Cu	Mo	Sr	Co
Cd	Liver	2,554.0	9,375.0	706.0	105.7	15.18	2.30	0.295	0.060
	Kidney	5,779.0	9,557.5	656.0	71.1	9.47	2.49	0.550	0.052
	Testicle	6,568.7	16,594.5	926.6	82.8	5.34	1.44	1.087	0.008
	Bone	5,106.6	–	2,310.0	101.5	6.89	0.18	34.080	0.000
Cd-2.5	Liver	2,263.0	9,466.3	691.0	154.6	17.90	2.35	0.475	0.058
	Kidney	5,252.0	10,803.2	685.0	102.6	13.03	2.59	0.375	0.051
	Testicle	5,977.7	16,364.2	1,048.9	99.7	6.14	0.87	1.530	0.113
	Bone	5,959.6	–	2,403.0	98.5	10.95	0.11	65.350	0.000
Cd-10	Liver	1,722.2	9,632.1	617.0	125.5	20.85	1.93	0.381	0.050
	Kidney	5,431.1	11,365.0	735.0	95.5	19.13	2.66	0.593	0.059
	Testicle	5,190.4	13,385.0	1,256.5	89.3	7.11	0.72	2.216	0.829
	Bone	5,786.3	–	2,675.0	97.9	6.71	0.08	46.280	0.000
Cd-75	Liver	2,508.2	10,936.2	729.0	120.0	19.00	2.68	0.296	0.056
	Kidney	5,842.1	12,048.2	801.0	89.3	20.15	2.03	0.580	0.073
	Testicle	5,272.3	13,575.1	999.6	101.0	7.58	1.40	3.085	0.260
	Bone	5,649.4	–	2,638.0	67.5	5.00	0.09	46.750	0.000
Cd-300	Liver	3,108.7	11,578.3	708.8	95.5	17.64	2.18	0.135	0.066
	Kidney	6,690.4	12,976.1	925.0	82.5	18.84	0.86	0.562	0.017
	Testicle	5,292.2	16,035.2	952.9	92.0	5.84	1.82	2.705	0.461
	Bone	5,994.1	–	2,566.0	68.2	4.21	0.00	34.080	0.000
Cd-600	Liver	3,146.0	11,885.5	772.0	150.0	22.15	2.47	0.156	0.068
	Kidney	7,169.1	13,700.6	976.7	94.1	35.85	1.35	0.684	0.007
	Testicle	3,567.3	5,068.1	218.4	173.0	14.85	3.25	26.750	1.115
	Bone	7,246.2	–	2,638.0	98.6	8.65	0.06	48.200	0.000

Table 7

Cadmium concentrations measured in the blood of broiler chickens exposed to cadmium load for maximum 42 days

Groups	Cd (μg/g dry matter)
Cd-0	< 0
Cd-0	< 0
Cd-2.5	< 0
Cd-2.5	< 0
Cd-10	0.13
Cd-10	0.17
Cd-75	0.22
Cd-75	0.29
Cd-75	0.13
Cd-75	0.23
Cd-75	0.28
Cd-75	0.24
Cd-300	0.30
Cd-300	0.28

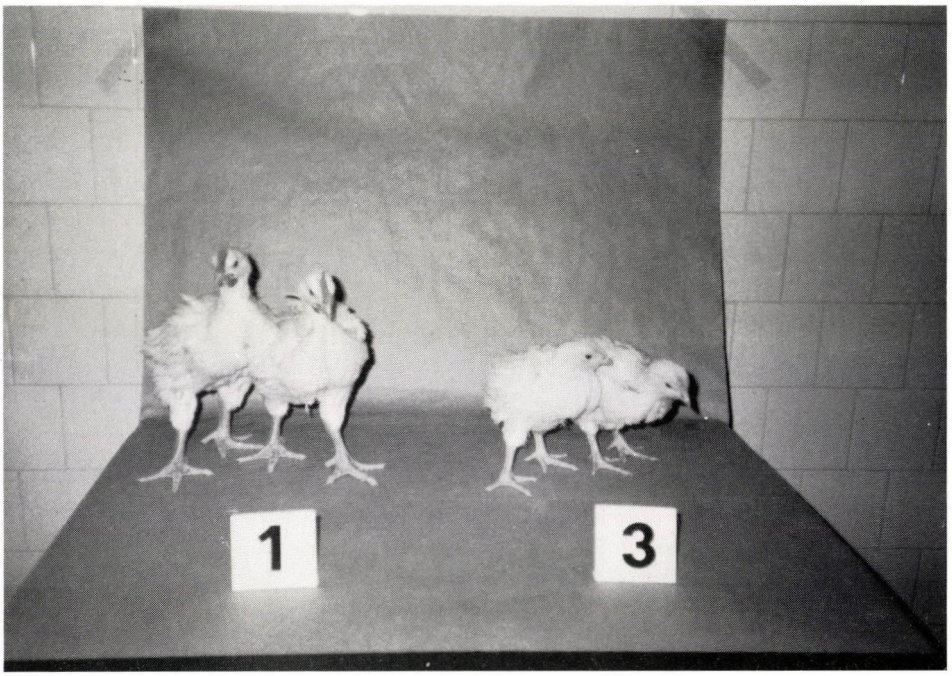


Fig. 1. Five-week-old control chickens weighing 1,050 g (1) and 5-week-old chickens fed 600 ppm Cd for 14 days, weighing 360 g (3)

Evaluation of the results, conclusions

Clinical signs. With the exception of chickens of groups Cd-30 and Cd-600, the treated chickens did not show pathological clinical symptoms in the first two weeks of treatment. They feathered and grew relatively well. Subsequently, their appetite gradually decreased, they showed growth retardation (Fig. 1), lustreless feathers, and several of them became diarrhoeic.



Fig. 2. Cockerels fed 75 ppm Cd up to 239 days of age, weighing approx. 4,000 g (1) and 7-week-old healthy cockerels weighing 2,070 g (2)

Mortality. The following mortalities occurred in the different groups up to 8 weeks of age: 2 chickens died in the control group, 1 in Cd-2.5, 1 in Cd-10, 10 in Cd-30, 0 in Cd-75, 1 in Cd-300, and 18 in Cd-600 (total number of deaths: 33). It should be noted that chickens of Cd-30, treated with an aqueous solution of Cd, developed symptoms of complete anorexia, rapid emaciation, huddling and diarrhoea, and died already at day 8–12 of the trial, in a matter of one or two days. The same applied to chickens fed 600 ppm Cd (group Cd-600), which died among similar symptoms before day 28 of the trial. This indicates that a daily Cd dose of 30 mg administered into the crop as an aqueous solution for 10 days was toxic to poultry, in the same way as the approx. 40–50 mg Cd per day fed for

21–27 days. The latter results show good agreement with the data of Pritzl et al. (1974), who reported that for poultry the LD_{50} of dietary Cd was 565 ppm.

Body mass. The results of weighing (Table 4) indicate that the body mass of chickens fed a Cd-supplemented diet either decreased markedly in proportion to the Cd load (groups Cd-30 and Cd-600) or was stagnant (group Cd-300). The only exception was group Cd-2.5 (which had an initial body mass slightly higher than that of the controls), the average body mass of which exceeded that of the control by max. 41.0 g even at the end of week 8.

The 10 cockerels fed 75 ppm Cd (Cd-75) for an additional 150 days (for 239 days altogether) showed a temporary weight loss but grew well thereafter. Four chickens in this group died among symptoms of arthritis, coprostasis and circulatory failure, but the rest remained symptomless throughout and reached a body mass of 3.8–4.3 kg (Fig. 2).

Feed conversion ratio. The feed conversion ratio was satisfactory in the control group (2.1 kg) and in group Cd-2.5 (2.4 kg). In the other groups, it was higher than that (max. 3.1 kg) and could not be evaluated in a realistic manner because of the rapid change in group size and due to the birds' decreased appetite.

Activities of digestive enzymes. (a) In the intestinal content, the activity of amylase (control: 1.29 U/mg protein; Cd-600: 0.59 U/mg protein), lipase (control: 36.75 ± 6.42; Cd-600: 19.89 ± 2.14 mU/mg protein) and trypsin (control: 56.02 ± 9.83; Cd-600: 15.91 ± 3.41 mU/mg protein) decreased significantly in group Cd-600, while total proteolytic activity in groups Cd-75, Cd-300 and Cd-600, as compared to the untreated control group. (b) In the pancreas homogenate, the activity of amylase significantly increased in groups Cd-300 (+164%) and Cd-600 (+171%), that of lipase in group Cd-600 (+127%), while that of trypsin in groups Cd-75 (+44%), Cd-300 (+106%), and Cd-600 (+32%). Total proteolytic activity significantly decreased in chickens of all three Cd-treated groups (–32, –43, –71%). The results show that Cd load exerts a significant influence on the activity of pancreatic hydrolases both in the intestinal content and in the pancreas (Tables 8 and 9). The activity reduction found in the intestinal content and the activity increase observed in the pancreas can probably be explained by a reduction of secretion; however, a direct influence of high Cd concentration cannot be ruled out either (Kósa et al., 1995).

Gross pathology. Necropsy of the two control chickens that had died revealed renal degeneration in one and chronic purulent inflammation of the tarsal joint accompanied by generalized amyloidosis in the other bird. Other chickens that died or were killed in the different groups at the times indicated above showed emaciation of different severity, depending on the degree of Cd load, accompanied by acute or chronic changes.

Table 8

Changes in the activity of four enzymes (amylase, lipase, trypsin and protease) in the pancreas as a result of cadmium load

Group		Pancreas			
		Amylase (E/mg	Lipase mE/mg protein	Trypsin mE/mg	Protease mE/mg
Cd-0 (Control)	x:	12.20	63.22	78.89	359.95
	sd:	± 1.47	± 17.32	± 13.55	± 70.83
	%:	100	100	100	100
Cd-2.5	x:	13.03	36.25	71.62	359.18
	sd:	± 2.44	± 5.01	± 8.87	± 40.40
	%:	109.01	57.33	98.70	99.78
Cd-10	x:	15.22	33.06	72.60	236.46
	sd:	± 2.98	± 4.96	± 16.50	± 40.40
	%:	125.81	52.29	92.02	65.69
Cd-75	x:	14.22	43.25	113.81	244.92
	sd:	± 3.56	± 15.73	± 13.18	± 42.08
	%:	116.55	68.41	144.26	68.04
Cd-300	x:	32.23	69.97	133.73	204.85
	sd:	± 7.53	± 11.28	± 41.39	± 40.12
	%:	264.18	110.67	206. 08	56.91
Cd-600	x:	33.10	143.60	104.75	139.15
	sd:	± 4.80	± 15.27	± 8.55	± 13.49
	%:	271.31	227.14	132.77	38.65

Emaciated cockerels in group Cd-30 had a swollen, dark red liver which was easy to tear and had indistinct structure (liver dystrophy). The testicles were atrophic, the kidneys pale brown and swollen (renal degeneration). The left ventricle of the heart was dilated and showed myocardial hypertrophy.

In groups Cd-75 and Cd-300 an emaciation of varying severity occurred, which was proportional to the dose of Cd and the duration of Cd exposure. In addition, there was degeneration of the liver and kidney, atrophy of the pancreas, spleen and testicles, left ventricle hypertrophy accompanied by a constriction of the lumen of ventricles. Most chickens examined after prolonged Cd load exhibited a dilatation of the glandular stomach and gizzard (Fig. 3), an extenuation of the gizzard wall and a thickening of its keratinoid layer (hyperkeratosis), and atrophy of the mucous membrane in the glandular stomach. These changes were consistent with those described by Pritzl et al. (1974). A large amount of mucus accumulated in the lumen of the glandular stomach. Some cockerels showed dilatation and chronic catarrhal inflammation of the crop. The tubular bones were easier to cut

through and contained a pale red bone marrow. In several chickens exposed to 42-day Cd load the kidney was of pale red colour and its substance was harder to tear (renal cirrhosis).

Table 9

Changes in the activity of four enzymes (amylase, lipase, trypsin and protease) in the intestinal content as a result of cadmium load

Group		Intestinal content			
		Amylase (E/mg	Lipase mE/mg protein	Trypsin mE/mg	Protease mE/mg
Cd-0 (Control)	x:	1.29	36.75	56.02	554.50
	sd:	± 0.39	± 6.42	± 9.83	± 91.25
	%:	100	100	100	100
Cd-2.5	x:	1.15	34.11	52.04	414.84
	sd:	± 0.40	± 3.47	± 10.46	± 66.28
	%:	89.00	92.81	92.89	78.81
Cd-10	x:	1.22	32.37	32.41	328.05
	sd:	± 0.28	± 4.23	± 7.43	± 40.04
	%:	94.57	88.08	57.85	59.16
Cd-75	x:	1.26	30.24	23.74	279.23
	sd:	± 0.39	± 3.90	± 5.75	± 42.66
	%:	97.67	82.28	42.37	50.32
Cd-300	x:	0.96	26.04	16.88	249.71
	sd:	± 0.13	± 1.87	± 3.54	± 24.90
	%:	74.41	70.85	30.13	45.03
Cd-600	x:	0.59	19.85	15.91	195.58
	sd:	± 0.10	± 2.14	± 3.41	± 8.69
	%:	45.73	54.01	28.40	35.27

In two cockerels exposed to a relatively low (2.5 mg Cd/kg body mass/day) but prolonged Cd load (which cockerels grew very well and were killed on day 68 of treatment), the testicles were found to be strikingly enlarged at necropsy (Fig. 4). The testicles measured and weighed 7 to 8 times as much as those of the control birds; they were soft to the touch, had a tight capsule and a bright and succulent cut surface.

At necropsy of cockerels fed 75 ppm Cd in the diet for more than 200 days, the liver, kidney and myocardium showed fatty infiltration (degeneration) and the tubular bones could be cut through more easily.

Histopathology. The organs of control birds (Cd-0) were found to have intact structure. In the treated groups, lesions of similar nature were observed, the

severity of which increased parallel to the dose of Cd. In almost all groups exposed to Cd, the *kidney* of chickens showed more or less severe tubulonephrosis. The tubular epithelium had become partially desquamated and the epithelial cells vacuolated (Fig. 5). The nuclei of some epithelial cells contained eosinophilic, inclusion body like formations. The tubular epithelium showed signs of regeneration. The mesangium cells of the glomerulus showed proliferation, and the glomerulus had become ischaemic (Fig. 5).

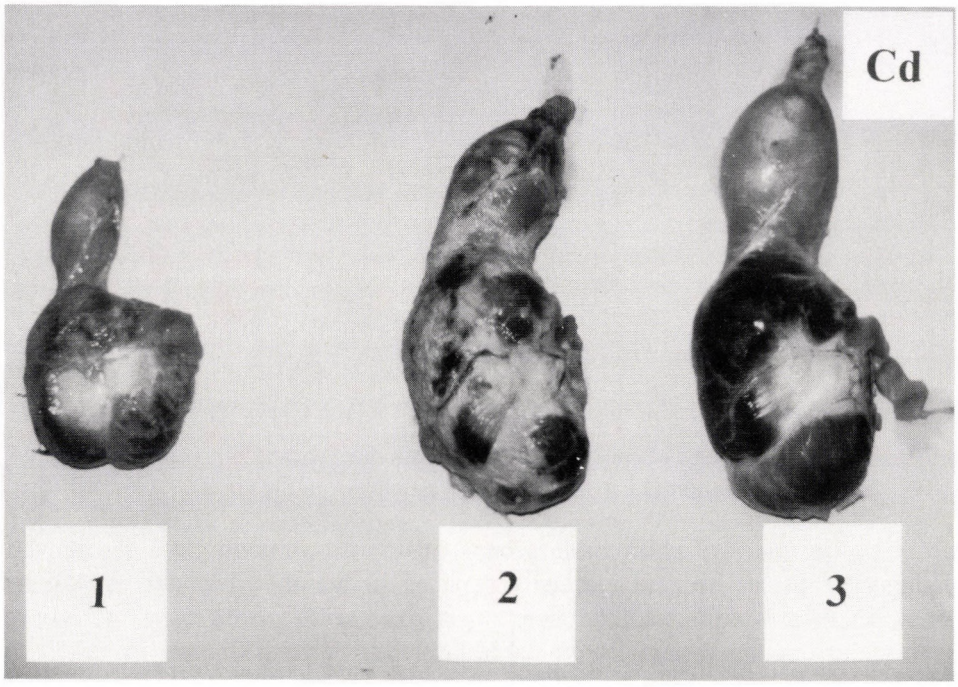


Fig. 3. Dilated glandular stomach and gizzard of cockerels treated for 68 days with a daily dose of 10 mg Cd administered as aqueous solution (2) and of those fed a diet containing 75 ppm Cd (3), as compared to the control birds (1)

In groups exposed to a high Cd load (75, 300 and 600 mg/kg of feed), the renal glomeruli were atrophic and the lumen of tubules contained hyaline cylinders (hyalin-droplet tubulonephrosis). Among the atrophic tubules a proliferation of connective tissue (fibrosis) was seen. The wall of arteries running in the renal interstices had become thickened while their lumen constricted.

In the *liver* of birds of all experimental groups a solitary hepatocyte necrosis was observed, the severity of which increased parallel to the dose of Cd. The nuclei of some hepatocytes contained eosinophilic, inclusion-like formations. Biliary duct proliferation of moderate degree also developed.

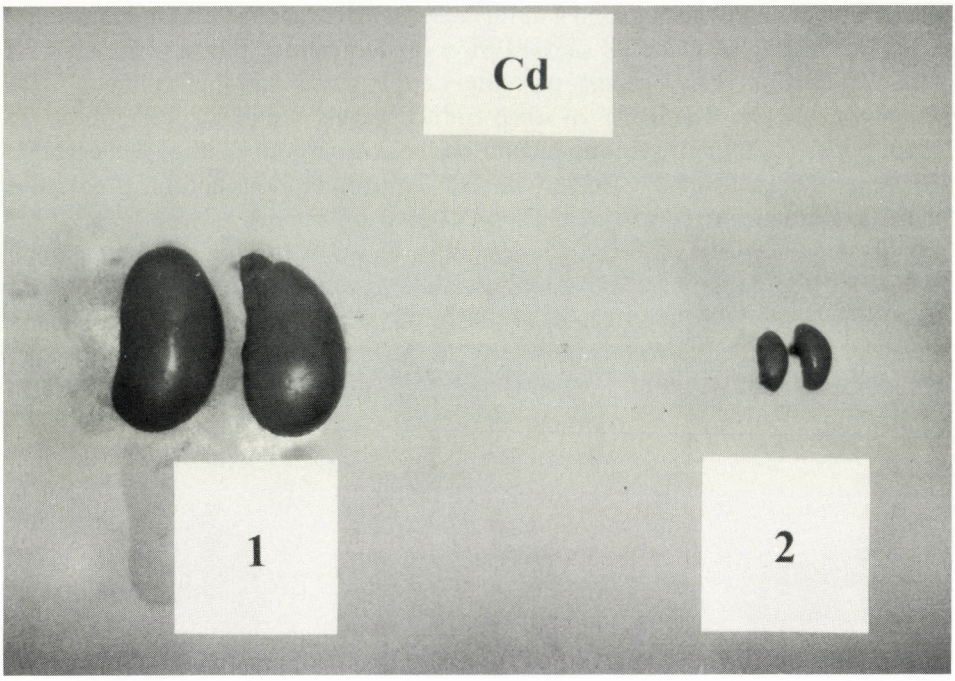


Fig. 4. Enlarged testicles of cockerels treated for 68 days with 2.5 mg Cd administered as aqueous solution (1), as compared to testicles from the control birds (2)

In the *spleen* of chickens in groups Cd-75, Cd-300 and Cd-600, the Malpighian corpuscles became markedly depleted in lymphocytes, and among the reticulocytes cell debris could be seen.

Injury of the germinal epithelium developed in the *testicles* of chickens in all Cd-treated groups. The markedly enlarged testicles of chickens exposed to a lower Cd load (2.5 and 10 mg Cd/kg b.m./day) showed a loosened tissue structure (oedema), a depletion of spermatogenic cells as compared to the control (Fig. 6) and the presence of large numbers of degenerated and detached cells among the spermatogenic cells (Fig. 7). The testicles of cockerels treated with higher doses of Cd (75, 300 and 600 mg/kg of feed) underwent severe atrophy. In the seminiferous tubules only Sertoli's cells could be seen. In some cases fibrosis developed in the interstices of the testicles (Fig. 8).

In the *brain*, there was perivascular oedema of varying severity. In addition to this, in the brain matrix of birds exposed to a heavier Cd load several homogeneously staining areas of indistinct structure (malacia) were seen, accompanied by necrobiosis among the nerve cells and moderate gliosis in the malacic areas.

The *myocardium* and the *skeletal muscles* showed mild interstitial oedema in chickens of groups Cd-2.5 and Cd-10. Exposure to a heavier Cd load resulted in

oedema and atrophy of the muscle fibres and focal nonreactive necrosis of the myocardium.

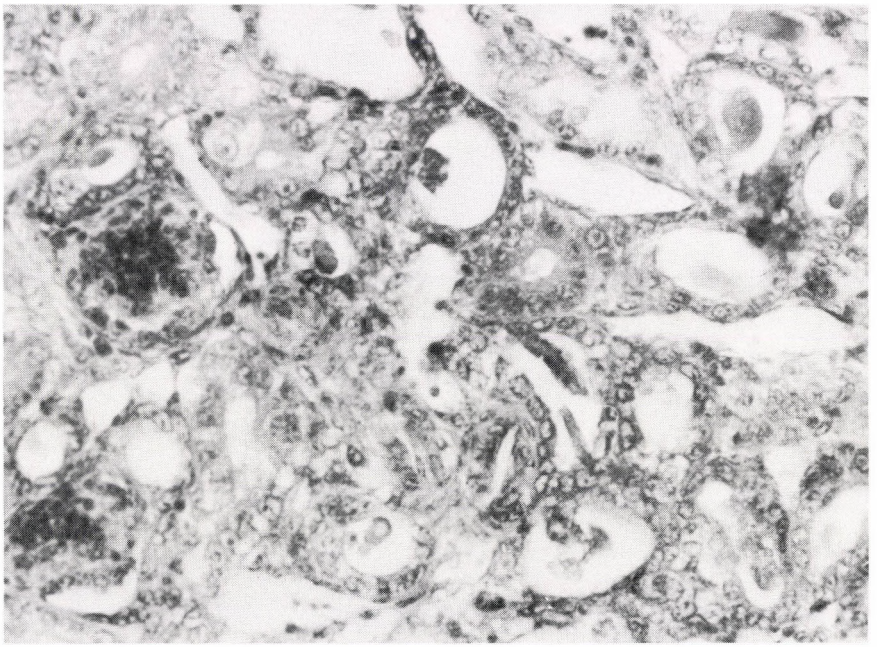


Fig. 5. Tubulonephrosis accompanied by regeneration in the kidney of a cockerel treated with 300 mg Cd/kg of feed for 42 days. Haematoxylin and eosin (H.-E.), × 250

In the *lungs* of chickens of all experimental groups it could be observed that the lumen of parabronchi had become dilated and contained a mucinous substance. Some of the atria became constricted or occluded and the smooth muscles in the wall of parabronchi had undergone hypertrophy. The air capillaries were unevenly dilated; in some areas the wall of air and blood capillaries had thinned down and ruptured (Fig. 9). In the lungs, the wall of arteries and arterioles became hypertrophic.

In the glandular stomach, some of the glandular epithelial cells of the lamina propria became detached (desquamated) and necrotic, while others had undergone atrophy. Already in chickens treated for 20–22 days, the epithelial cells of the glands located in the *gizzard* mucosa became atrophic and their lumen was filled out by an eosinophilic substance (keratinoid). In the Cd-treated chickens that substance covered the mucosa more abundantly than in the controls. Some of the atrophic glands became completely necrotic and a connective tissue composed of collagenic fibres was seen proliferating in the place of the glands. The gizzard of

chickens treated with Cd for several weeks contained glands showing severe atrophy, while the lamina propria exhibited signs of fibrosis.

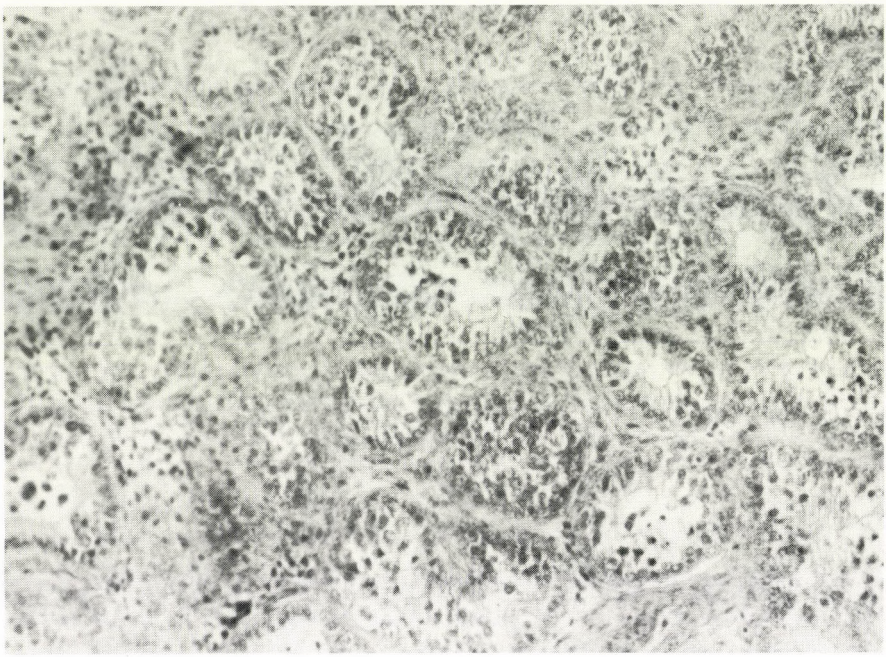


Fig. 6. Testicle from a 42-day-old untreated control cockerel. H.-E., × 125

It could be observed already in chicken groups treated with lower doses of Cd (Cd-2.5, Cd-10, Cd-75) that the villi of the small intestinal mucosa became shortened and distorted, and fusion of several intestinal villi could be seen. In the lamina propria of fused, irregular villi there was a proliferation of connective tissue consisting of collagenic fibres (Fig. 10).

In chickens from groups exposed to a higher Cd load, the epithelial cells of the exocrine glands of the *pancreas* showed a depletion of eosinophilic granules in the apical part of the cells, vacuolation of the cytoplasm, and atrophy. A mild atrophy of the islets of Langerhans developed only in the groups exposed to the heaviest Cd load.

In chickens of group Cd-2.5, the distal epiphysis of the *femur* showed no change as compared to the control. In birds of group Cd-10, the epiphysis exhibited lesions indicative of calcification disturbances. The epiphyseal cartilage was degenerated, its calcification zone broadened and became uneven. The bone trabeculae were irregular and numerous degenerated osteoblasts were seen on them. In animals exposed to a high Cd load, severe osteoporosis, osteoclastic osteolysis, and

bone marrow aplasia developed; the severity of these lesions depended on the dose of Cd administered (Figs 11 and 12).

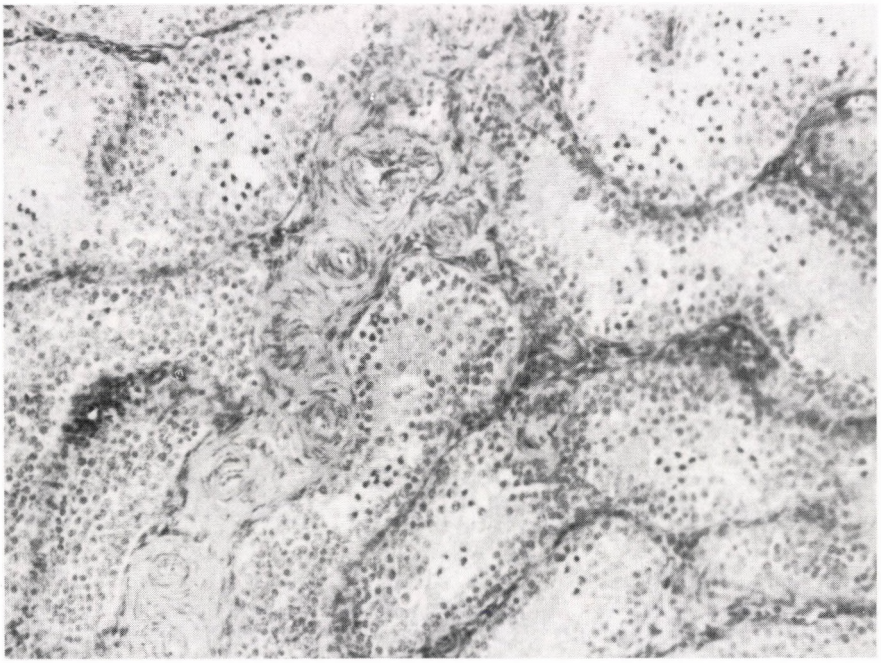


Fig. 7. Testicle from a cockerel treated with 10 mg Cd/kg body mass for 42 days. Note proliferation in the tunica media cells of blood vessels running in the interstices of testicles, resulting in vasoconstriction. Disorganization and oedema of the germinal epithelial cells and appearance of an eosinophilic substance in the lumen of the seminiferous tubules. H.-E., × 125

In the organs of chickens treated with Cd over a long period (for more than 200 days) similar light microscopic changes developed, accompanied by connective tissue proliferation in several organs. Osteoporosis seen in the bone-tissue was accompanied by myeloid hyperplasia of the bone marrow as a sign of regeneration.

Transmission electron microscopy (TEM) was performed on organ (liver, pancreas, myocardium, kidney, brain) samples taken from two control chickens and six chickens exposed to a high Cd load (Cd-75, Cd-300 and Cd-600). The organs of birds from all treated groups showed practically similar lesions, i.e. glycogen depletion in the cytoplasm of hepatocytes and aggregation of the chromatin substance in their nuclei in the form of inclusion-like formations. The mitochondria were swollen, their structure was indistinct, and some of the cristae became fragmented. A depletion and fragmentation of the granular endoplasmic reticulum

(RER) was seen, with detachment of ribosomes (degranulation) from its surface (Fig. 13). The amount of smooth endoplasmic reticulum (SER) increased. Cells showing signs of necrobiosis could be observed among the hepatocytes (Fig. 14).

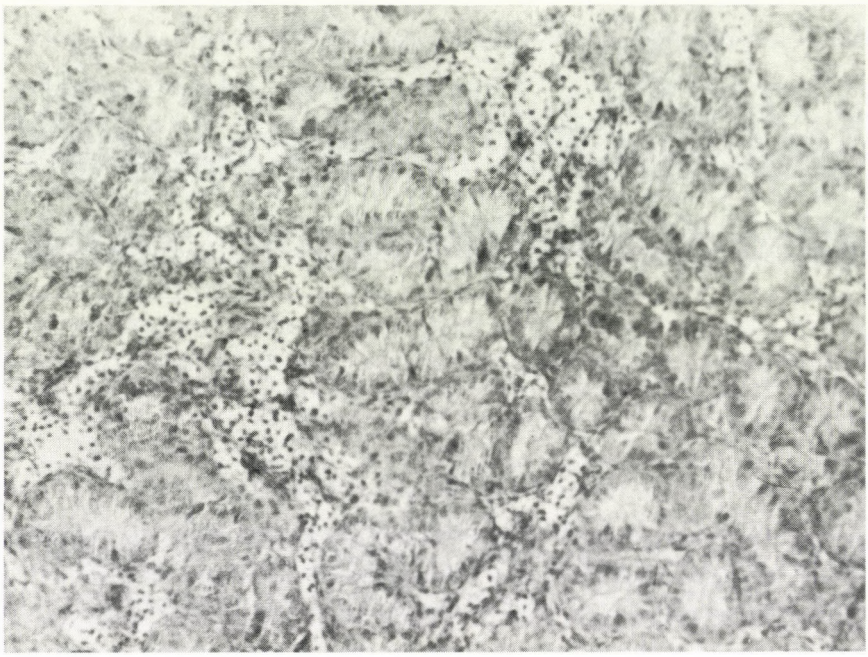


Fig. 8. Testicle from a cockerel fed 300 ppm Cd for 42 days. Note degeneration (dystrophy and atrophy) of the germinal epithelium. H-E., × 125

As compared to the control, the amount of granules in the cytoplasm of glandular epithelial cells of the pancreas had decreased. The cisternae of the RER had become dilated and partial necroses and autophagous vacuoles developed in the cytoplasm (Fig. 15).

Histopathological examination of the myocardium revealed cyst-like dilatation of the sarcoplasmic reticulum, cellular and interstitial oedema, formation of megamitochondria with the reduplication of cristae (Fig. 16). The cytoplasm of the tubular epithelial cells of the kidney contained autophagous vacuoles and hyalin droplets, the basal striation became indistinct and the mitochondria degenerated. The nucleus of tubular epithelial cells contained inclusion-like bodies. The basal membrane of the glomeruli thickened, its structure became indistinct, and the pedicels of podocytes coalesced.

In the neurons of the cortical substance of the brain the ribosomes aggregated and the cisternae of the RER became dilated and fragmented. In the cyto-

plasm of these neurons dense granules appeared, while in their nuclei the chromatin substance showed signs of degeneration.

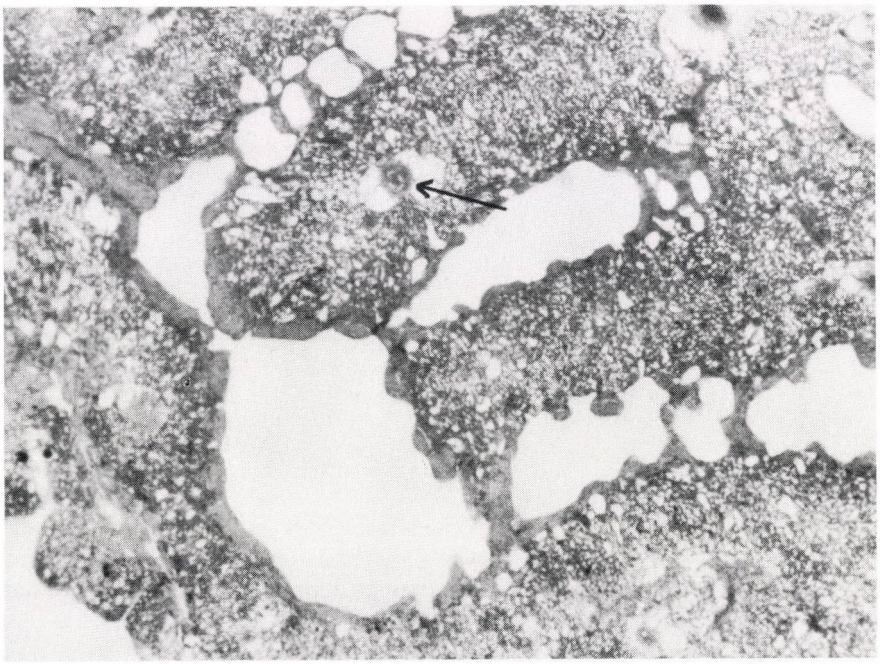


Fig. 9. Lungs from a cockerel fed 600 ppm Cd for 28 days. Note pulmonary emphysema accompanied by hypertrophy of the muscle layer of parabronchi. Proliferation of the tunica media of interstitial arteries, resulting in constriction of the blood vessel lumen. H.-E., × 50

Summing up the clinical and morphological findings, it can be established that the prolonged administration of even small amounts of Cd (2.5 mg Cd/kg b.m./day) caused kidney, liver and testicular damage in cockerels. It also damaged the mucous membrane of the digestive tract (glandular stomach, gizzard and small intestine). A higher Cd load produced lesions, besides the above organs, also in the myocardium, spleen, bone-tissue and bone marrow. In addition to the direct cytotoxic effect of Cd, malabsorption and maldigestion due to changes developing in the digestive tract may also have played a role in the development of the clinical signs and pathological lesions observed.

Chemical assays. From the mineral element concentrations determined by chemical assay of the organs examined the following conclusions can be drawn. As a result of the Cd load, the concentration of Cd increased in all organs in a degree depending on the Cd dose administered.

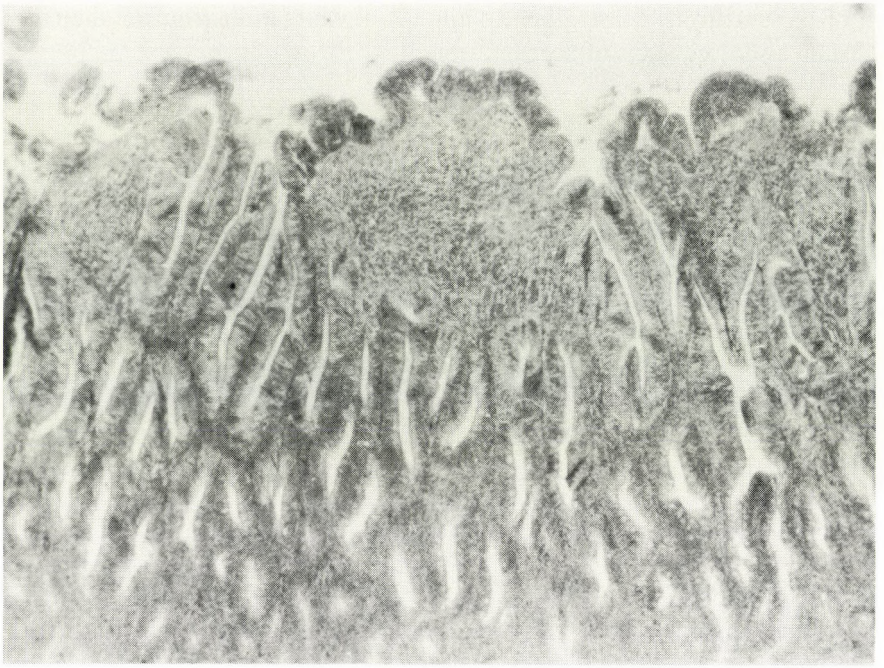


Fig. 10. Small intestine from a cockerel fed 75 mg Cd for 42 days. Note atrophy and fusion of the intestinal villi. H.-E., × 50

Average Cd concentration of the *liver* increased substantially (by 2–3 orders of magnitude). In group Cd-2.5 it reached a level (48.23 mg/kg) almost 400 times higher while in group Cd-300 a concentration (667.80 mg/kg) approx. 4,000 times higher than the control value (0.15 mg/kg). These results indicate that the liver is one of the principal sites of Cd accumulation. These values are higher than those reported by Leach et al. (1979) and Sharma et al. (1979).

A similarly marked increase occurred in the Cd content of the *kidney*, from an average of 0.11 mg/kg measured in control chickens to a maximum of 333.0 mg/kg in the group administered the highest dose of Cd (an increase by three orders of magnitude). This value is lower than the maximum concentration measured in chickens (616–2718 mg/kg dry matter) exposed to a lower Cd load. At the same time, it roughly corresponds to the concentrations measured by Leach et al. (1979).

The Cd load resulted in an accumulation of Cd in the *skeletal muscles* and *myocardium*. In the latter, the highest concentration (11.25 mg/kg) was found in group Cd-600.

The Cd concentration of the *brain* increased to 1.33 mg/kg in the group exposed to the highest Cd load.

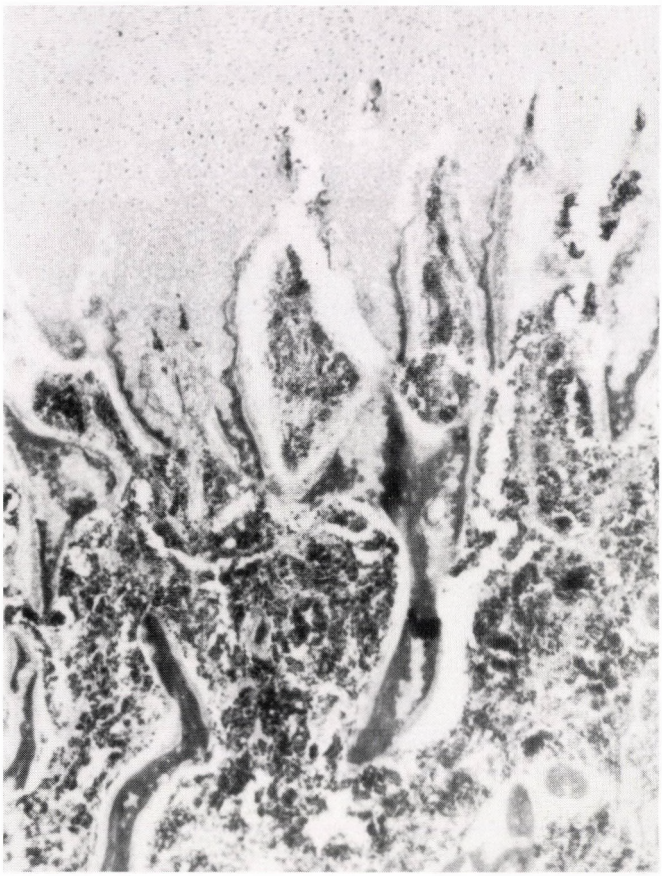


Fig. 11. Tibiotarsal bone from a 42-day-old untreated control cockerel. H.-E., × 50

A very substantial Cd accumulation was measured in the *spleen* and *testicles*, with maximum concentrations reaching 29.57 and 17.50 mg/kg, respectively. A similarly big increase of Cd concentration was found in the *pancreas*: the maximum (83.86 mg/kg) was measured in group Cd-300.

The Cd concentration of the *lungs* was also high (max. 34.48 mg/kg).

A somewhat less expressed increase occurred in the Cd content of the *bones* (maximum concentration: 4.66 mg/kg).

The Cd concentration of *blood* samples rose parallel to the Cd load, and reached its maximum (0.30 μg/g dry matter) in the group designated Cd-300.

As regards changes in the concentrations of other *microelements* during Cd load, the concentration of *Cu* increased primarily in the kidney and testicles. In the kidney it increased to a level (35.85 mg/kg) approx. four times higher than that found in the controls. The concentration of *Zn* hardly changed in the kidney but

appreciably increased in the testicles during medium Cd load and notably decreased in the bones (e.g. in group Cd-300).

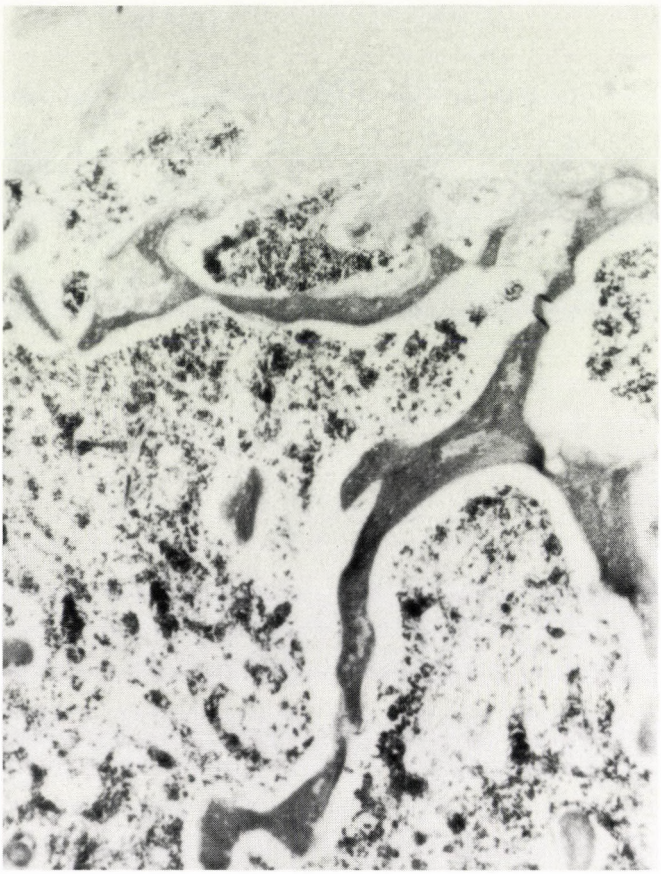


Fig. 12. Tibiotarsal bone from a 42-day-old cockerel fed 300 ppm Cd for 42 days. Severe osteoporosis and bone marrow aplasia can be seen. H.-E., × 50

During Cd load, the concentration of *Fe* markedly increased in the spleen and, in some cases, the testicles. Similar findings were obtained for *Mn*, the concentration of which showed an especially marked rise in the testicles, reaching 12.50 mg/kg. At the same time, the Mn concentration of the bones slightly decreased.

In chickens exposed to a high Cd load, the concentration of *Mo* decreased in the kidney and even more in the bones but markedly increased in the testicles (maximum value: 3.25 mg/kg). No changes could be measured in the concentration of *Se*. The concentration of *B* multiplied in the testicles and especially in the pancreas.

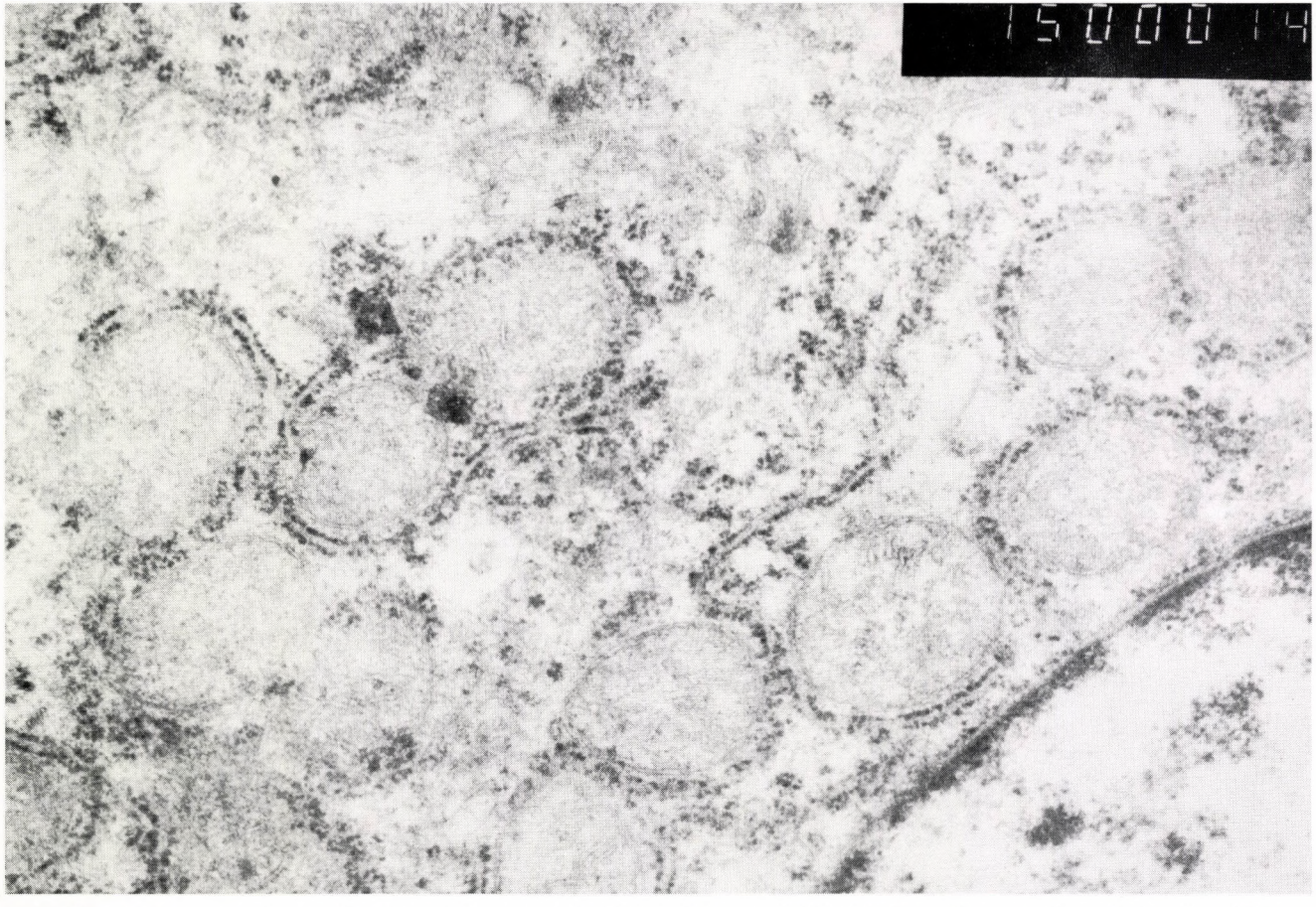


Fig. 13. Electron micrograph of a hepatocyte from a cockerel fed 600 ppm Cd for 28 days. Degeneration of mitochondria can be seen, accompanied by fragmentation and degranulation of the RER. × 36,000

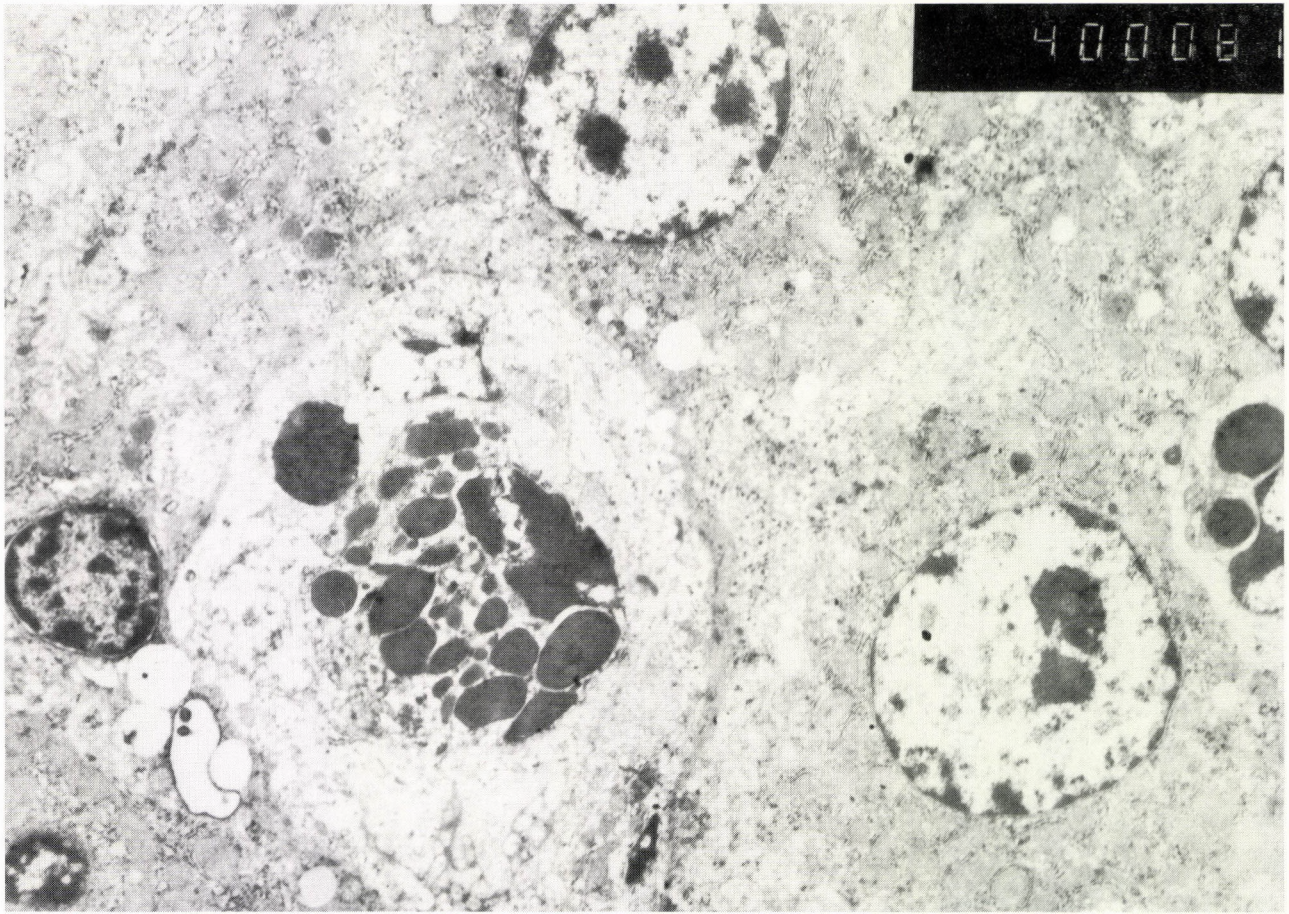


Fig. 14. Electron micrograph of a hepatocyte from a cockerel fed 75 mg Cd for 42 days. Solitary hepatocyte necrosis. × 8,800

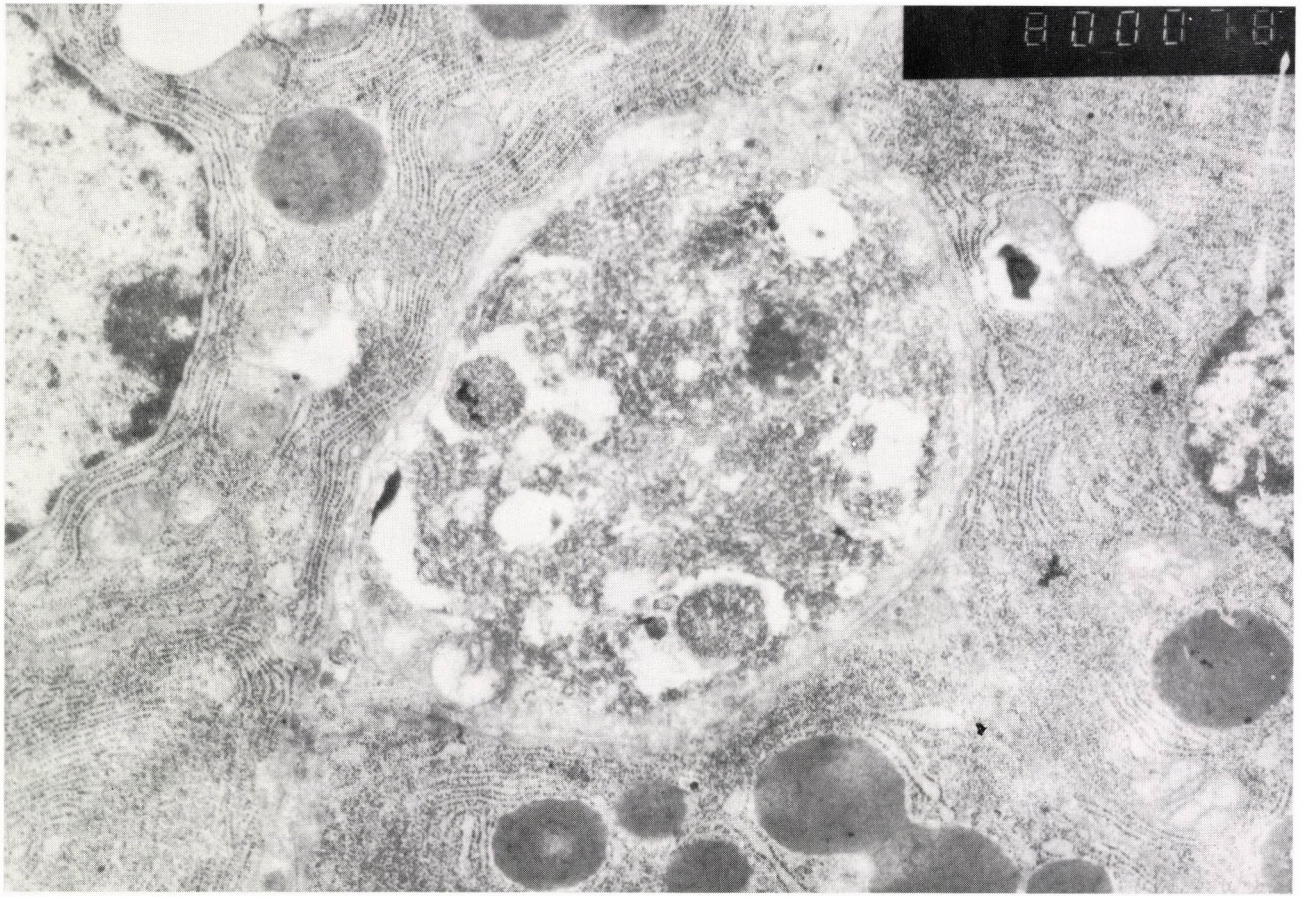


Fig. 15. Electron micrograph of the pancreas from a cockerel fed 75 ppm Cd for 42 days. Note degeneration of a part of the cell. × 17,000

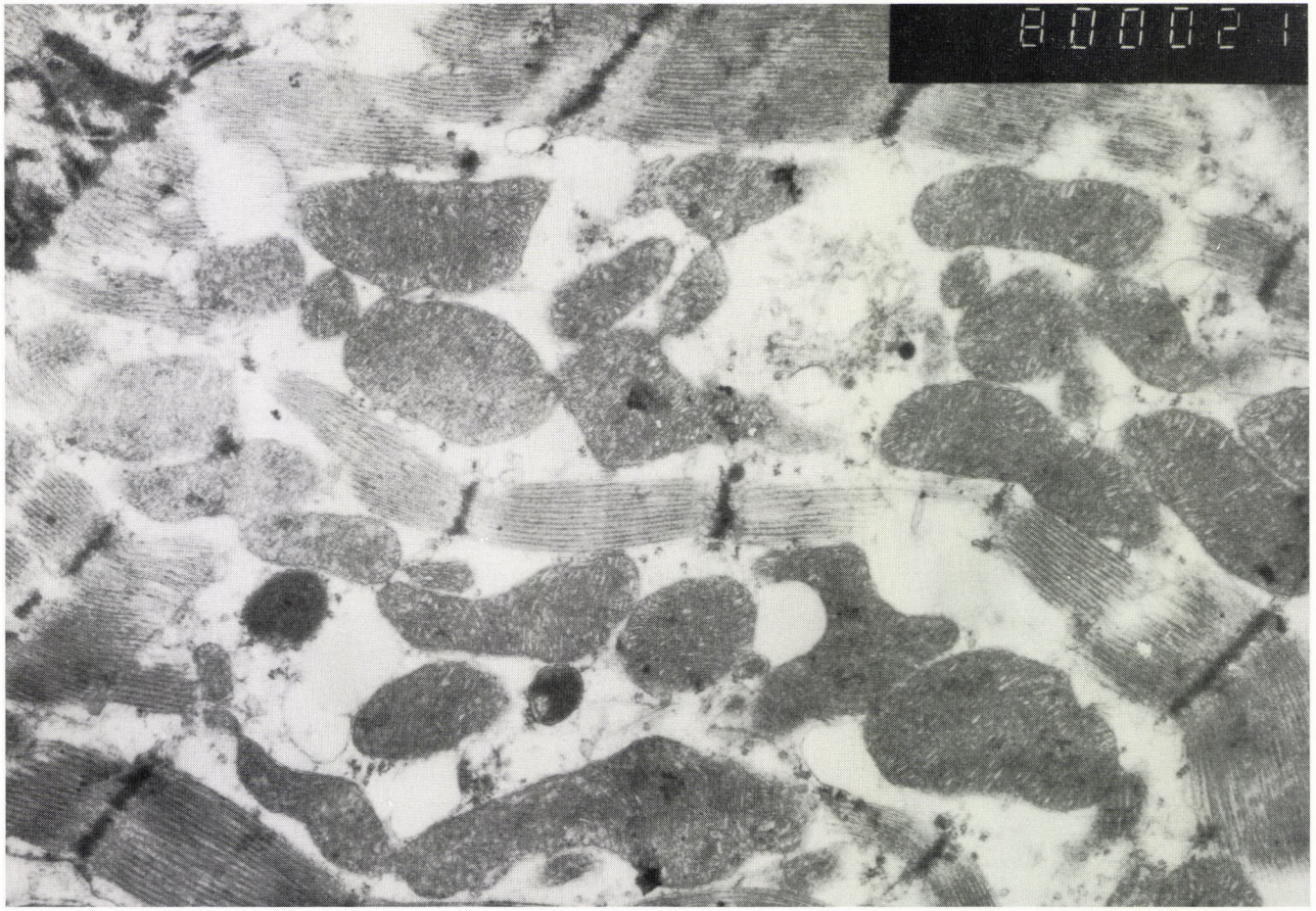


Fig. 16. Electron micrograph of the myocardium from a cockerel fed 600 ppm Cd for 28 days. Note the formation of megamitochondria. × 19,200

During Cd load, the concentration of *Sr* slightly decreased in the liver and increased by an order of magnitude in the testicles. The *Co* content of the testicles also rose markedly. The results confirm that Cd exerts a positive or negative influence on the metabolism of several microelements (Cu, Zn, Fe, Mn, Mo, B, Sr).

Of the *macroelements*, the concentration of *P* slightly increased in all organs except the spleen, testicles and lungs. It reached the highest level in the bones (65,772 mg/kg) and brain (14,002 mg/kg).

Although only slightly, the concentration of *Ca* increased in almost all organs upon Cd load. The only exception was the bone in which Ca content slightly decreased in chickens of group Cd-300 (104,457 mg/kg). At the same time, even its maximum value (125,507 mg/kg) was only moderately higher than the average measured in the control birds (116,604 mg/kg). From this it appears that in chickens the Cd load did not produce a substantial or consistent reduction in total Ca and P concentration of the bones. In this context, it should be mentioned that while the Zn, Mn and Cu content of the bones decreased in a dissimilar degree, with the exception of Cu their concentration did not markedly change in the other organs.

During Cd load, the biggest increase in the concentration of *Mg* occurred in the testicles (max. 1,256 mg/kg). The concentration of *K* increased in the liver (11,885 mg/kg) and kidney (13,700 mg/kg). The amount of *Na* increased in most organs but mainly in the bones, while in the testicles its concentration (3,567 mg/kg) dropped to nearly half the level found in the controls. Upon Cd load, the concentration of *S* more than doubled in the testicle (from 4,261 to a maximum of 10,943 mg/kg) while it hardly changed in the bones (maximum average value: 874.3 mg/kg).

Acknowledgements

The authors thank Dr. Emma Kósa for the determination of enzyme activities. This work was supported by the National Research Fund, project no. 1270.

References

Anke, M., Hennig, B., Groppel, B., Partschefeld, M. and Grün, M. (1978): The biochemical role of cadmium. In: Kirchgessner, M. (ed.) Trace Element Metabolism in Man and Animals. 3rd edn. Arbeitskreis Tierernährungssforschung, E. Weihenstephan, pp. 540–548.

Anke, M., Klinger, G. and Grün, M. (1982): The sex and age dependence of the cadmium concentration in human beings and animals. In: Anke, M. (ed.) Mengen and Spurenelemente. Karl-Marx-Universität, Leipzig. pp. 103–113.

Black, F., Anke, M. and Schneider, H. J. (1979): Kadmium Symposium. Wissensch. Beitrage, Friedrich-Schiller Univ., Jena.

Bokori, J. and Fekete, S. (1995): Complex study of the physiological role of cadmium. I. Cadmium and its physiological role. Acta Vet. Hung. **43,** 3–43.

Bokori, J., Fekete, S., Kádár, I., Vetési, F. and Albert, M. (1993): Complex study of the physiological role of aluminium. II. Aluminium tolerance tests in broiler chickens. Acta Vet. Hung. **41,** 235–264.

Bruckner, Cl. (1988): Der Einfluss verschiedener Futterzusatzstoffe auf die Cadmium-Retention in der Niere beim Hühnerküken. Thesis, Munich.

Bunker, V. W., Lawsob, S. M., Belves, T. H. and Clayton, E. B. (1984): The intake and excretion of lead and cadmium by the elderly. Am. J. Clin. Nutr. **39,** 803–808.

Fassett, D. W. (1972): Cadmium. In: Lee, D. H. K. (ed.) Metallic Contaminants and Human Health. Academic Press, New York. p. 97.

Flick, D. F., Kraybill, F. H. and Dimitroff, M. J. (1971): Toxic effects of cadmium: A review. Environm. Res. **4,** 71.

Fox, M. R. S. (1974): Effect of essential minerals on cadmium toxicity. A review. J. Food Sci. **39,** 321–324.

Freeland, J. H. and Cousins, R. J. (1973): Effect of dietary cadmium on anaemia, iron absorption and Cd binding protein in the chickens. Nutr. Rep. Internat. **8,** 337.

Frieden, E. (1985): New perspectives on the essential trace elements. J. Chem. Educ. **62,** 917.

Hapke, J. H. (1975): Toxicologie für Veterinärmediziner. Ferdinand Enke Verlag, Stuttgart.

Hapke, J. H. (1992): Auf Tiere einwirkende toxische Umweltnoxen. Züchtungskunde **64,** 262–270.

Holcman, A. and Smodis, B. (1973): Heavy metal content in hen's eggs. In: Anke, M., Meissner, D. and Mills, F. C. (eds) Trace Elements in Man and Animals. Tema 8. Verlag Media Touristic, Jena 1, 1993, 1249.

Kósa, E., Szabó, J., Fekete, S. and Kővári, L. (1995): Reports of the Hungarian Academy of Sciences. Budapest, January 23, 1995.

Leach, R. M., Wang, L. W. K. and Baker, D. E. (1979): Cadmium and the foodchain. The effect of dietary Cd on tissue composition in chicks and laying hens. J. Nutr. **109,** 437–443.

Pais, I. (1989): Importance of Microelements for Life (in Hungarian). University of Horticulture and Food, Budapest.

Pritzl, C. M., Lie, H. Y., Kienholz, W. E. and Whiteman, E. C. (1974): The effect of dietary cadmium on development of young chickens. Poultry Sci. **53,** 2026–2029.

Régius-Mőcsényi, Á., Anke, M., Kronemann, H. and Szentmihályi, S. (1985): Cadmium content and influence of cadmium contamination in plants and animals. In: Pais, I. (ed.) New Results in the Research of Hardly Known Trace Elements. Proc. Internat. Symp. Univ. Horticult., Budapest.

Sas, B. and Tu, P. V. (1989): Detection and effects of food-contaminating toxic elements, with especial regard to some toxicological and biochemical consequences of cadmium load (in Hungarian). Publication of the Environmental Protection Conference of the Hungarian Association for Agricultural Science. Budapest, 1989.

Sharma, P. R., Street, C. J., Verma, P. M. and Shupe, K. L. J. (1979): Cadmium uptake with feed and its distribution to food products of livestock. Environm. Health Perspect. **28,** 59–66.

Stadelman, J. W. and Prati, E. D. (1989): World's Poultry Science Journal **45,** 247–246. In: Anke, M., Meissner, D. and Mills, F. C. (eds) Trace Elements in Man and Animals. Tema 8. Verl. Media Touristik, Jena.

Stolley, U. (1989): Der Einfluss verschiedener Vitamine und Spurenelemente auf die Cadmium-retention in der Niere beim Hühnerküken. Thesis, Munich.

Sumino, K., Hayakawa, K., Shibata, T. and Kitamura, S. (1975): Heavy metals in normal Japanese quail tissues. Arch. Environm. Health **30,** 487–507.

Ueberschär, H. K., Vogt, H., Nezel, K. und Matthes, S. (1982): Verminderung der Cadmium-Toxizität bei Broilern durch Selenzusätze zum Futter. Arch. Geflügelk. **46,** 9–13.

Washko, P. H. and Cousins, J. R. (1976): Metabolism of 109 Cd in rats fed normal and low-calcium diets. J. Toxic. Environm. Health **1,** 1055–1066.

Acta Veterinaria Hungarica 43 (2–3) pp. 229–246 (1995)

EFFECT OF AVOPARCIN ON RUMEN FERMENTATION AND DUODENAL NUTRIENT FLOW IN SHEEP

Hedvig FÉBEL[1], A. ROMVÁRY[2], Ildikó ZSOLNAI-HARCZI[1] and Szilvia HUSZÁR[1]

[1]Department of Physiology, Research Institute for Animal Breeding and Nutrition, H–2053 Herceghalom, Hungary; [2]Cyanamid Co., A–1120 Wien, Austria

(Received November 16, 1994)

In a digestive-physiological experiment series, the effect exerted by avoparcin on rumen fermentation and on the ruminal digestion of nutrients was studied in wethers provided with rumen and duodenal cannulas, as a function of the composition of feed as substrate. Three control (I, II, III) groups containing different amounts of rumen degradable protein (RDP) and nonstructural carbohydrate (NSC) were formed (composition of diet: group I, 74% RDP and 38% NSC; group II, 57% RDP and 32% NSC; group III, 48% RDP and 23% NSC). The feeding of control diets was followed by the administration of experimental diets containing avoparcin (groups I+A, II+A, and III+A). The dose of avoparcin was 0.75 mg/kg body weight. Irrespective of the RDP and NSC content of the feed, avoparcin reduced the molar ratio of acetic acid and increased that of propionic acid, decreased the acetic acid / propionic acid ratio, and increased the molar ratio of isobutyric acid. Ammonia concentration of the ruminal fluid was significantly lower in group I+A than in the corresponding control group (I). Avoparcin supplementation of diet III improved the apparent digestibility of organic matter from 52.9% to 56.4%. When added to a ration of high NSC and RDP content (I), avoparcin decreased the true digestibility of organic matter from 77.0 to 72.5%. Compared to diet III as well as to diets II and III, avoparcin significantly increased the ruminal degradation of cellulose and hemicellulose, respectively. Avoparcin supplementation of the diet significantly decreased the microbial N content of the duodenal chymus irrespective of the NSC and RDP content of the diet. In group I+A, the amount of dietary N passed from the rumen into the duodenum in 24 h was significantly higher (7.1 g/day vs. 2.7 g/day). In wethers fed the diet of the lowest NSC and RDP content (III), avoparcin supplementation (III+A) increased the apparent digestibility of N in the rumen. In contrast, in wethers fed diets of higher RDP and NSC content (I and II) the true ruminal digestibility of N decreased. Irrespective of the RDP and NSC content of the diet, avoparcin supplementation significantly reduced the efficiency of microbial protein synthesis. The enhanced propionic fermentation induced by the administration of avoparcin allows more efficient utilization of the dietary energy. The higher ratio of undegraded, bypass protein reaching the duodenum provides the animal with a protein source degraded and utilized directly in the small intestine. The results support the observation that avoparcin increases the body weight gain of animals during fattening.

0236–6290/95/$ 4.00 © 1995 *Akadémiai Kiadó, Budapest*

Key words: Avoparcin, wether, rumen fermentation, nutrient, duodenal flow

In ruminant animals, the utilization of nutrients is closely related to the activity of microorganisms. For the host organism it is clearly beneficial if feed constituents undegradable by its own digestive enzymes and therefore utilized poorly or not at all are made use of by the microorganisms. At the same time, it is detrimental if proteins of high biological value and carbohydrates well hydrolyzable by pancreatic and intestinal enzymes "fall victim" to microbial degradation which involves a loss of energy. In addition, microbial degradation occurring in the rumen yields by-products (CH_4, CO_2, NH_3, heat) whose departure involves energy or nitrogen loss for the host animal.

Based upon these considerations, two opportunities present themselves for influencing rumen fermentation by chemical means: either the balance of fermentative microorganisms shall be changed so as to reduce the loss at digestion to the minimum, or the biologically valuable feed constituents shall be protected from degradation in the rumen.

The number of substances serving for the manipulation of rumen fermentation is very large. The effect of these "rumen manipulators" is usually not specific, as they can simultaneously affect several components of the fermentative and degradation processes occurring in the rumen (Chalupa, 1984). Of the "rumen manipulators", growth-promoting antibiotics are used the most widely, as they exert an effect not only on carbohydrate fermentation but also on protein metabolism of the rumen.

Of compounds belonging to the group of growth promotants, the biggest amount of experimental knowledge is available on ionophore antibiotics, especially on monensin and lasalocid. However, other non-ionophore antibiotics also possess a growth-promoting effect. Such a substance is the glycopeptide compound avoparcin (Avotan 100®, Cyanamid Co., U.K.), produced by the fungus *Streptomyces candidus*. Like other glycopeptide-type antibiotics (e.g. aridicin and actaplanin), avoparcin inhibits the incorporation of N-acetyl-glucosamine into peptidoglycan, which is a building material of bacterial cell walls (Speth et al., 1981). Avoparcin exerts a selective effect on rumen microorganisms. It inhibits the growth of most Gram-positive microorganisms, including that of the cellulolytic *Ruminococcus* spp. *Bacteroides succinogenes*, which produces succinate (the precursor compound of propionic acid), is more resistant to the effect of avoparcin than *Ruminococcus* strains, but less resistant than Gram-negative bacteria (Stewart and Duncan, 1985).

Based upon research results obtained in recent years, avoparcin is acquiring increasing importance in the nutrition of farm animals. It improves the feed conversion efficiency and body weight gain of fattening pigs (Roth and Kirchgessner,

1975; Herold et al., 1984; Günther, 1987), feedlot cattle (Johnson et al., 1979; Dyer et al., 1980), and sheep (Szelényi et al., 1989).

From earlier research results it is known that the administration of avoparcin favourably influences the energy metabolism of the rumen through the enhancement of propionic fermentation (Johnson et al., 1979; Dyer et al., 1980; Cottyn et al., 1983). At the same time, only few and sometimes conflicting data are available on the role that avoparcin plays in ruminal N metabolism. This may partly be due to the fact that the composition of the feed as substrate may substantially modify the results of experiments. A further uncertainty involved in the digestive-physiological study of avoparcin was that the positive or negative effects observed in the experiments were difficult to explain. This was mainly due to the fact that the experiments were usually performed on rumen-fistulized animals or in an *in vitro* system, which provided data exclusively on the changes occurring in the rumen. These experimental systems, however, failed to provide sufficient information on the quantity of nutrients degraded in the rumen and passing into the duodenum.

In order to resolve the above contradictions and to elucidate the role of avoparcin in the fermentative and digestive processes of the rumen, digestive-physiological experiments were conducted in which the effect exerted by avoparcin on rumen fermentation and ruminal digestion was studied *in vivo*, on sheep fitted up with ruminal and duodenal cannulas, as a function of the composition of the ration.

Materials and methods

Eight mature wethers (average body weight: 62 kg) of the Hungarian Merino breed were used in the experiments. The wethers had previously been subjected to surgery and fitted up with a Jarret's rumen fistula (Hecker, 1974) inserted into the dorsal sac of the rumen, and with a simple T-shaped fistula (Aguilar and Depeters, 1988) inserted into the duodenum at a site approx. 10 cm caudal to the pylorus. After the recovery period (about 1 month) the animals were placed into individual slatted-floor pens with self-waterers, with drinking water and salt licks being available *ad libitum*.

The effects of the feed and the applied growth promotant were studied in a multifactorial (growth promotants, diets) experiment of incomplete block design. For studying the effects of avoparcin, three different control (I, II, III) diets of different nonstructural carbohydrate (NSC) and rumen degradable protein (RDP) content were formulated (control groups: group I, fed a ration of 38% NSC and 74% RDP content; group II, fed a ration of 32% NSC and 57% RDP content; group III, fed a ration of 23% NSC and 48% RDP content).

The control diets were fed for a period of 18 days, which comprised a 13-day prefeeding phase followed by a 5-day sample collection phase. Group I in-

cluded 2, while groups II and III comprised 3 wethers each. After the control phase, the same animals (self-controlled arrangement) were fed the above rations supplemented with avoparcin (A) (experimental groups: group I+A, fed a ration of 38% NSC and 74% RDP content supplemented with avoparcin at a dose rate of 0.75 mg/kg body weight; group II+A, fed a ration of 32% NSC and 57% RDP content + 0.75 mg/bwkg avoparcin; group III+A, fed a ration of 23% NSC and 48% RDP content + 0.75 mg/bwkg avoparcin).

The arrangement described above corresponded to phase 1 of the experiment. This was followed by phase 2, which was practically a repetition of phase 1, with the experimental animals being assigned to a different group. Thus, in phase 2 of the experiment groups I and II included 3 animals each, while group III consisted of 2 wethers.

The composition and nutrient content of the diets fed in the experiments are shown in Table 1.

The ruminal degradability of dietary protein was determined by an *in sacco* method (Ørskov and McDonald, 1979). NSC content was calculated from the accurate nutrient content of the basic rations using the following formula (Stokes et al., 1991): 100 – [crude protein + (NDF – NDF-bound crude protein) + crude fat + crude ash]. The nutrient content values presented in Table 1 correspond to the results obtained by analytical methods described in detail later on, while the NSC value has been calculated by the above formula.

The quantity of dry matter to be taken up by the animals was set at 60 g dry matter/bwkg$^{0.75}$ (Van Es and Van Der Meer, 1980). That quantity was administered to the animals in two equal portions, at 8:00 a.m. and at 4:00 p.m.

The 13-day prefeeding period was followed by a 5-day test sample collection phase (Fig. 1). The duodenal nutrient flow was determined by the so-called double marker method (Faichney, 1980). Starting on day 6 of the trial, 12 g Cr-NDF/kg of feed/day was added to the feed as particlemarker and 0.25 g polyethylene glycol (PEG)/bwkg/day was added to it to label the liquid phase of digesta. The Cr-NDF marker was prepared by the method of Udén et al. (1980).

Between day 14 and 16 of the experiment, duodenal samples (approximately 200 ml on each occasion) were collected according to the schedule shown in Fig. 1, at 6-hour intervals, bringing forward the start of collection by 4 hours every day. Using that schedule, a total of 12 samples were collected in 3 days. After collection, the samples were weighed, poured into a nylon bag and stored frozen at –20 °C. The samples collected from individual animals were processed for the analyses as follows: after thawing, the samples collected at the specific time-points were adjusted to identical weight, then pooled and homogenized. This pooled sample constituted a representative sample collected in 24 h. When using the two-phase marker method, half of the sample was lyophilized according to the specified criteria, and the other half was centrifuged at 30,000 × *g* for

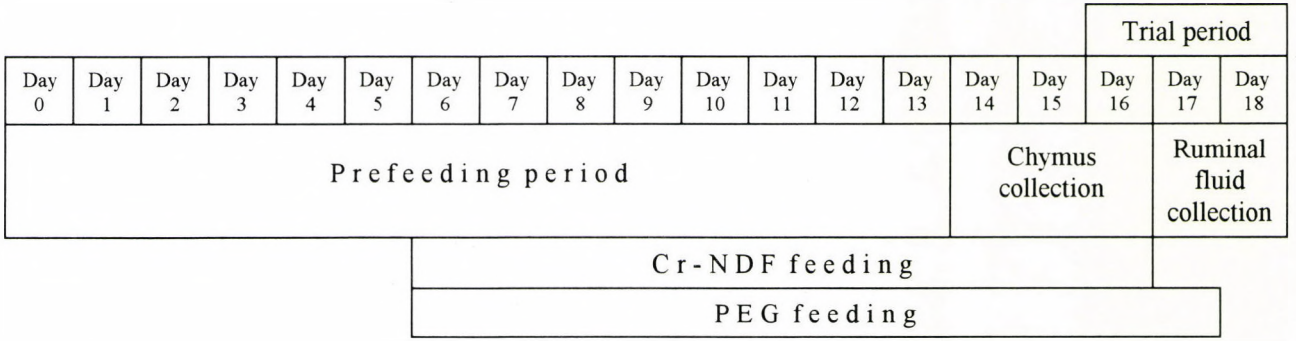
20 min. The resulting pellet was also lyophilized. The samples were ground through a sieve of 1 mm pore size before used for analysis.

Table 1

Composition and nutrient content of diets fed in the experiment

Constituent	Diet I	Diet II	Diet III
Composition, %			
Meadow hay	30.00	50.00	70.00
Corn meal	44.23	23.50	17.25
Extracted sunflower	24.00	8.00	–
Corn gluten	–	7.50	12.25
Barley	–	9.44	–
Limestone	1.27	1.06	–
Premix	0.50	0.50	0.50
Nutrient content, g/kg DM			
Organic matter	932.9	932.7	936.3
Crude protein	181.3	177.0	177.5
NDF	359.7	438.6	508.0
ADF	178.7	220.4	271.5
Cellulose	147.7	178.3	222.5
Hemicellulose	191.1	218.2	236.5
Nonstructural carbohydrate (NSC)	381.9	317.2	233.4
Rumen degradable protein (RDP), %	74	57	48
NSC : RDP ratio	2.85	3.14	2.74

The dry matter, crude ash and Kjeldahl-N contents of feed and duodenal samples were determined according to the standards specified for the determination of the nutritive value of feeds (Hungarian Standard MSz 6830). NDF and ADF content was measured by the method of Van Soest and Robertson (1985), while cellulose content by a modification of the procedure described by Crampton and Maynard (1938). For the determination of ammonia content the Berthelot reaction was used (Chaney and Marbach, 1962). For determination of their amino acid content the chymus samples were hydrolyzed with 6N hydrochloric acid at 110 °C for 24 h. Amino acids were separated in an AMINOCHROM II type instrument by ion-exchange column chromatography. A method based on purine content determination was applied for identifying the portion of bacterial origin within the total N of the duodenal content (Zinn and Owens, 1986). For measuring the Cr content of chymus samples in an ARL 3410 ICP spectrometer the samples were processed by the method of Christian and Coup (1954). Polyethylene glycol was quantitated by a turbidimetric method described by Hyden (1955).



Schedule of chymus collection

Day 14					5:00				11:00				17:00					23:00
Day 15			3:00				9:00				15:00				21:00			
Day 16	1:00					7:00				13:00				19:00				

Schedule of rumen fluid collection (pH, volatile fatty acid, ammonia and urea)

Day 17	11:30	19:30
Day 18	11:30	

Schedule of rumen fluid collection (separation of bacteria)

Day 18	7:30	11:30	14:30

Fig. 1. Schedule of the experiment

Samples from the ruminal fluid were taken on days 17 and 18 of the trial, 3 h after feeding (at the times specified in Fig. 1). The pH of the samples was immediately measured (PHM-27 pH meter, Radiometer, Copenhagen, Denmark) and recorded. To determine the volatile fatty acids, the samples were precipitated with 25% metaphosphoric acid, centrifuged and then stored frozen (Supelco Inc., 1975). The volatile fatty acids were determined on an SP-1200/1% H_3PO_4 on 80/100 Chromosorb WAW filled column. The concentrations of ammonia and urea were measured by the so-called Berthelot reaction using phenyl-hypochlorite reagent. Ruminal volume and the dilution rate were determined by the method of Dehareng and Godeau (1989).

For the separation of bacteria, ruminal fluid samples were taken on day 18 of the trial, before feeding and 3 and 6 hours thereafter. The ruminal fluid was first subjected to low-speed centrifugation (1000 × *g*) at 4 °C for 20 min, then the bacteria were separated from the supernatant by multiple high-speed centrifugation cycles. The bacterial pellet was deep-frozen and lyophilized. Lyophilized bacterial samples were assayed for dry matter, crude ash, N and purine content by the methods described above.

Comparison of the results obtained for the control and experimental groups and their statistical evaluation were performed using multifactorial analysis of variance and *t* test (Sváb, 1981).

Results

The changes induced by avoparcin in rumen fermentation 3 h after feeding are summarized in Table 2.

As compared to the value measured in group II, the ration supplemented with avoparcin (II+A) resulted in a significantly higher pH of the ruminal fluid. No such difference between the control and test ration was observed in the other groups.

Avoparcin supplementation of the rations fed to groups I and II caused a significant decrease of the total volatile fatty acid concentration of the ruminal fluid from 132.72 to 117.84 mmol/l and from 135.28 to 112.63 mmol/l, respectively.

When studying the individual volatile fatty acids, the biggest changes were found in the molar ratio of acetic acid and propionic acid, in the relative ratio of these two volatile fatty acids, and in the quantity of isobutyric acid. Irrespective of the RDP and NSC content of the diet, the molar ratio of acetic acid decreased, that of propionic acid increased, the acetic acid:propionic acid ratio decreased, and the molar ratio of isobutyric acid rose as a result of avoparcin supplementation. In the molar ratio of butyric acid, avoparcin supplementation caused a significant reduction (from 10.40% to 8.56%) in one case, when added to the ration of the lowest NSC and RDP content (III). A higher isovaleric acid content was measured in the

ruminal fluid samples taken from wethers of group II+A. A reduction in the molar ratio of valeric acid was observed for ruminal fluid samples taken from wethers fed an avoparcin-supplemented ration of the highest NSC and RDP content (I+A).

As compared to the diet of the highest RDP and NSC content (I), avoparcin supplementation (I+A) significantly lowered the ammonia concentration of the ruminal fluid (25.04 vs. 16.52 mmol/l). At the same time, avoparcin supplementation of rations II and III failed to cause a significant change in the ammonia content of the ruminal fluid. Avoparcin supplementation caused no significant difference in the urea concentration of the rumen.

The data obtained on the dilution rate and on rumen volume are also shown in Table 2. Avoparcin administration did not give rise to significant changes in these parameters.

The changes caused by avoparcin supplementation in the duodenal nutrient flow, in the ruminal degradation of nutrients and in the N metabolism of the rumen, as well as other relevant results of the experiment are presented in Tables 3–6.

Avoparcin supplementation did not affect the duodenal flow and the apparent ruminal digestibility of dry matter (Table 3). No significant difference was found in the passage of organic matter to the duodenum on avoparcin administration. Avoparcin supplementation (III+A) of the ration of the lowest NSC and RDP content (III) improved the apparent digestibility of organic matter from 52.9% to 56.4%. When added to the ration of the highest NSC and RDP content (I), avoparcin (I+A) decreased the true digestibility of organic matter from 77.0% to 72.5%.

Avoparcin supplementation (II+A, III+A) of rations of lower NSC and RDP content (II and III) exerted a significant influence on the ruminal degradation of different fibre fractions (Table 4): it significantly improved the ruminal degradability of cellulose as compared to diet III, and that of hemicellulose as compared to diets II and III. Avoparcin supplementation of diet I caused a reduction in the ruminal digestibility of ADF and cellulose.

Results on ruminal N metabolism indicate that while avoparcin did not affect the rate of passage of total N and non-ammonia N, it significantly lowered the microbial N content of the duodenal chymus irrespective of the NSC and RDP content of the diet (Table 5). At the same time, when added to diet I, avoparcin significantly increased (from 2.7 g/day to 7.1 g/day) the quantity of feed N passing from the rumen into the duodenum in 24 h. When added to the ration of the lowest NSC and RDP content (III), avoparcin (III+A) improved the apparent ruminal digestibility of N. At the same time, when added to diets of higher RDP and NSC content (I and II), avoparcin exerted an opposite effect, as the true ruminal digestibility of N decreased upon its administration. Irrespective of the RDP and NSC content of the diet, the efficiency of microbial protein synthesis was lower in animals fed avoparcin than in the control groups (19.6, 18.2 and 20.2 g N/kg

Table 2

Effect of avoparcin on some parameters of the ruminal fluid

Item	I	I+A	II	II+A	III	III+A
pH	5.63	5.63	5.73	5.94**	5.95	6.01
	±0.18	±0.19	±0.13	±0.20	±0.24	±0.18
Total volatile fatty acids, mmol/l	132.72	117.84*	135.28	112.63***	108.80	105.24
	±15.47	±16.90	±17.50	±13.66	±10.85	±11.92
C2, mol%	60.71	48.12***	62.44	54.97***	68.44	61.89***
	±2.09	±2.48	±1.46	±2.95	±1.80	±2.31
C3, mol%	21.31	33.45***	20.68	27.42***	16.67	24.88***
	±2.06	±3.43	±1.61	±5.85	±1.64	±2.06
C2:C3	2.89	1.46***	3.04	2.11***	4.15	2.51***
	±0.38	±0.21	±0.29	±0.52	±0.46	±0.32
iC4, mol%	2.73	4.49***	2.09	3.51***	1.96	2.52**
	±0.29	±0.54	±0.43	±0.52	±0.29	±0.59
C4, mol%	12.02	11.59	12.16	11.32	10.40	8.56***
	±1.24	±1.73	±1.56	±2.91	±0.96	±1.00
iC5, mol%	1.78	1.50	1.30	1.68**	1.40	1.26
	±0.54	±0.30	±0.23	±0.31	±0.36	±0.27
C5, mol%	1.45	0.91***	1.34	1.42	0.97	0.91
	±0.17	±0.28	±0.20	±0.54	±0.24	±0.24
Ammonia, mmol/l	25.04	16.52***	19.14	16.95	11.06	11.10
	±4.14	±5.35	±4.34	±4.60	±1.79	±4.82
Urea, mmol/l	11.06	9.49	9.05	9.97	4.76	5.10
	±2.02	±3.08	±2.68	±2.23	±1.08	±2.16
Dilution rate, %/h	7.56	7.04	9.95	8.01	8.22	6.75
	±1.58	±0.63	±1.08	±1.49	±1.91	±1.46
Rumen volume, l	7.34	7.45	7.46	7.80	9.06	11.43
	±1.60	±0.10	±0.90	±1.19	±0.85	±1.83

Significant difference exists between respective values of the control and the related experimental group at the following levels:
* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$

Table 3

Effect of avoparcin on the ruminal degradation of dry matter and organic matter

	I	I+A	II	II+A	III	III+A
Dry matter						
Intake,	1300.8	1301.5	1339.8	1300.5	1325.0	1330.5
g/day	±126.9	±119.5	±65.0	±121.4	±105.2	±84.8
Duodenal flow, g/day	601.4	598.0	692.3	645.3	739.5	696.5
	±41.4	±49.7	±50.3	±37.5	±30.2	±54.4
Apparent digestibility,	53.7	53.9	48.3	50.1	44.2	47.8
%	±3.8	±2.5	±3.1	±4.0	±2.9	±1.2
Organic matter						
Intake,	1210.0	1216.0	1253.0	1212.2	1244.0	1242.5
g/day	±117.4	±115.5	±60.7	±113.1	±98.0	±70.1
Duodenal flow, g/day	449.8	453.7	526.3	507.3	585.8	541.9
	±25.0	±38.1	±26.3	±25.0	±24.9	±33.8
Apparent digestibility,	62.8	62.6	57.9	58.2	52.9	56.4*
%	±2.7	±1.9	±1.9	±2.7	±2.6	±0.6
True digestibility,	77.0	72.5**	70.4	68.7	65.8	67.5
%	±2.1	±1.4	±3.4	±2.6	±2.5	±2.5

Significant difference exists between respective values of the control and the related experimental group at the following levels: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$

Table 4

Effect of avoparcin on the ruminal degradation of individual fibre fractions

	I	I+A	II	II+A	III	III+A
NDF						
Intake,	479.5	483.3	581.8	595.5	659.3	684.2
g/day	±46.5	±54.2	±38.9	±65.1	±49.3	±31.8
Duodenal flow, g/day	175.9	186.4	231.0	213.8	255.9	227.6*
	±13.9	±8.8	±10.3	±10.3	±17.1	±7.4
Digestibility,	62.9	61.2	60.2	64.3	61.4	66.7
%	±5.3	±2.9	±2.9	±3.4	±4.7	±1.6
ADF						
Intake,	242.4	233.8	296.3	298.0	356.1	377.9
g/day	±23.5	±18.3	±21.9	±29.9	±26.1	±19.5
Duodenal flow, g/day	105.5	112.1	135.4	133.4	155.5	146.3
	±9.1	±2.9	±5.6	±8.6	±10.1	±4.0
Digestibility,	56.1	51.9	54.0	55.1	56.4	61.2
%	±5.5	±2.9	±4.3	±2.3	±4.4	±2.3
Cellulose						
Intake,	198.4	194.6	239.7	242.3	290.3	309.9
g/day	±19.1	±16.5	±18.3	±27.2	±21.1	±12.8
Duodenal flow, g/day	79.9	84.9	101.3	96.8	112.5	103.8
	±7.2	±2.9	±7.4	±7.8	±9.1	±2.5
Digestibility,	59.3	56.1	57.5	59.9	61.2	66.5**
%	±5.9	±4.2	±5.1	±4.7	±1.7	±0.8
Hemicellulose						
Intake,	237.1	249.6	285.5	297.5	303.2	306.2
g/day	±23.0	±37.9	±17.1	±37.8	±23.1	±17.3
Duodenal flow, g/day	70.4	74.2	95.6	80.4**	100.4	81.4*
	±7.4	±6.9	±6.4	±7.5	±11.0	±5.3
Digestibility,	69.9	70.0	66.5	72.8**	66.7	73.4**
%	±5.7	±2.5	±2.1	±2.9	±2.6	±0.9

Significant difference exists between respective values of the control and the related experimental group at the following levels: * $P < 0.05$ and ** $P < 0.01$

Table 5

Effect of avoparcin on N metabolism and microbial protein synthesis in the rumen

	I	I+A	II	II+A	III	III+A
N						
Intake, g/day	38.7 ±3.8	36.7 ±2.8	38.3 ±1.8	37.0 ±3.5	37.5 ±2.9	38.2 ±2.5
Passage to the duodenum, g/day						
Total N, g/day	22.2 ±1.9	20.9 ±1.8	26.6 ±1.8	24.7 ±1.1	31.8 ±0.9	29.8 ±1.7
Non-ammonia N, g/day	20.7 ±1.7	19.5 ±1.6	25.2 ±1.3	23.4 ±0.9	30.6 ±0.9	28.7 ±1.7
Microbial N, g/day	17.9 ±1.4	12.4*** ±1.0	16.3 ±1.4	13.2** ±1.5	16.6 ±0.9	14.4* ±1.4
Bypass N, g/day	2.7 ±0.6	7.1*** ±0.7	9.1 ±1.2	10.3 ±1.2	14.0 ±0.7	14.3 ±1.1
Digestibility, %						
Apparent digestibility, %	42.7 ±4.5	42.6 ±2.8	30.6 ±3.5	33.1 ±2.4	15.3 ±1.3	22.2* ±2.9
True digestibility, %	89.0 ±1.1	76.7*** ±1.4	72.7 ±2.1	68.5* ±1.3	59.4 ±2.3	59.8 ±1.7
*Efficiency of microbial protein synthesis, g N/kg OMTD**	19.6 ±1.5	14.1*** ±1.2	18.2 ±0.9	15.9** ±0.9	20.2 ±1.4	17.0* ±1.4

*OMTD = organic matter truly digested in the stomachs

Significant difference exists between respective values of the control and the related experimental group at the following levels:
* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$

Table 6

Duodenal flow of different amino acids as influenced by the experimental treatments (g/day)

Amino acids	I	I+A	II	II+A	III	III+A
Total	114.7	106.3	136.7	126.9	162.9	152.9
	±9.4	±9.6	±9.9	±9.2	±8.9	±13.3
Essential[1]	51.4	46.8	57.6	55.5	69.2	65.8
	±3.9	±4.1	±3.2	±4.2	±5.2	±6.2
Nonessential[2]	63.2	59.5	79.1	71.3	93.7	87.1
	±5.8	±6.2	±6.9	±5.9	±4.2	±7.1
Arginine[1]	5.1	4.9	4.5	4.9	5.0	5.4
	±1.4	±0.7	±0.8	±1.2	±0.4	±0.7
Histidine[1]	3.1	2.8	2.5	3.3*	3.2	4.0
	±0.5	±0.3	±0.4	±0.5	±0.5	±0.7
Isoleucine[1]	5.1	4.2*	5.5	5.1	6.9	6.3
	±0.5	±0.5	±0.8	±0.9	±0.3	±0.8
Leucine[1]	10.6	9.8	16.3	14.3	20.4	18.6
	±1.7	±1.3	±2.3	±2.7	±2.1	±2.3
Lysine[1]	6.9	6.6	6.6	6.7	6.9	7.1
	±1.3	±0.3	±0.7	±1.6	±0.6	±1.2
Methionine[1]	2.2	2.4	2.3	2.5	2.7	2.2
	±0.6	±0.3	±0.3	±0.8	±0.3	±0.3
Phenylalanine[1]	6.3	5.4	7.1	6.3	8.4	7.6
	±1.5	±0.8	±0.8	±1.1	±1.3	±0.7
Threonine[1]	6.0	5.4	5.6	5.9	6.9	6.7
	±0.9	±0.3	±1.0	±0.9	±1.2	±0.9
Valine[1]	6.1	5.3	7.1	6.5	8.6	7.9
	±0.8	±0.7	±0.8	±1.3	±0.7	±0.9
Alanine[2]	8.3	8.1	12.6	10.4	16.2	13.5
	±1.2	±1.2	±2.2	±1.7	±1.3	±2.1
Aspartic acid[2]	12.3	11.4	13.1	11.9	15.4	14.1
	±1.5	±1.5	±1.8	±2.1	±1.4	±1.4
Glutamic acid[2]	20.9	19.1	29.4	24.9	34.8	32.6
	±3.0	±2.4	±3.3	±3.7	±3.6	±3.7
Glycine[2]	6.5	6.0	6.5	6.1	6.9	6.9
	±0.9	±0.5	±1.1	±1.2	±0.7	±1.3
Proline[2]	5.0	5.7	6.3	6.6	7.5	6.9
	±0.8	±1.1	±1.7	±0.8	±1.2	±0.7
Serine[2]	5.5	5.1	6.8	6.3	7.6	7.1
	±0.7	±0.5	±1.0	±1.1	±0.6	±0.8
Tyrosine[2]	4.7	3.9	4.4	5.0	5.3	5.9
	±1.3	±0.7	±0.9	±1.0	±0.8	±0.6

Significant difference exists between respective values of the control and the related experimental group at the level of * $P < 0.05$

OMTD for diets I, II and III, vs. 14.1, 15.9 and 17.0 g N/kg OMTD for avoparcin-supplemented diets I+A, II+A and III+A, respectively).

Like that of total N, duodenal flow of total, essential and nonessential amino acids did not change significantly following avoparcin administration (Table 6). Only in two cases did the analysis of individual amino acids reveal significant differences induced by avoparcin supplementation: the passage of histidine to the duodenum increased from 2.5 g/day (II) to 3.3 g/day (II+A), while that of isoleucine decreased from 5.1 g/day (I) to 4.2 g/day (I+A).

Discussion

Experimental data obtained in this study on ruminal fermentative processes indicate that avoparcin exerts a major effect on the molar ratio of volatile fatty acids. Consistently with the results obtained by other investigators (Ingle et al., 1978; Chalupa et al., 1981; Cottyn et al., 1983; Froetschel et al., 1983), in this experiment avoparcin lowered the molar ratio of acetic acid, raised that of propionic acid, and thus decreased the acetic acid : propionic acid ratio irrespective of the RDP and NSC content of the diet. At the same time, other researchers (Dyer et al., 1980; Darden et al., 1985; Unsworth et al., 1985) did not find differences in the concentration of the individual volatile fatty acids after avoparcin supplementation.

With the exception of one case, avoparcin supplementation did not affect the pH of the ruminal fluid in any of the experiments. The pH value of the ruminal fluid was 5.63, 5.73 and 5.95 in control groups I, II and III, respectively, while it was 5.63, 5.94 and 6.01 in the groups receiving avoparcin supplementation (I+A, II+A and III+A, respectively). Namely, 3 h after feeding the pH value of the ruminal fluid was at or below 6.0 in both the control and the experimental groups, which is highly favourable for propionic fermentation. The effect of avoparcin manifested itself in that even more propionic acid was produced at that pH value than when the three different control diets were fed. Increased propionic fermentation allows a more efficient utilization of the dietary energy by decreasing methane and carbon dioxide production resulting in a loss of energy. In addition, it enables more propionic acid to participate in the process of gluconeogenesis, thereby preventing the use of amino acids as a source of energy and making available a larger quantity of amino acids for protein synthesis in the tissues. In ruminants, these favourable changes induced in the energy and protein metabolism may have importance not only during fattening but also in milk production.

Although in ruminal fluid samples taken 3 h after feeding the pH values were similar, in the total volatile fatty acid concentration a significant decrease was observed. On avoparcin administration, the total volatile fatty acid concentration of the ruminal fluid was significantly lower than in control groups I and II. A similar result, i.e. a decrease of the total volatile fatty acid content, was reported by Froetschel et al. (1983).

The decrease found in total volatile fatty acid concentration may be explained by the results obtained on ruminal digestion and especially those on the efficiency of microbial synthesis which was found to decrease upon avoparcin administration. This result shows that avoparcin induces a change in the rumen which reduces the intensity of microbial growth. This is supported by the results obtained on ruminal N metabolism, namely that avoparcin administration caused a significant reduction in the passage of microbial N to the duodenum, irrespective of the RDP and NSC content of the diet. When ration I was fed, the duodenal flow of undegraded (bypass) feed N was significantly higher at a lower microbial N content. The true ruminal digestibility of N simultaneously underwent a significant decrease (from 89.0% to 76.7%). Probably this is why a lower ammonia concentration was measured in the rumen.

When diet II was supplemented with avoparcin, the true ruminal digestibility of N decreased (from 72.7% to 68.5%); however, that decrease was not as expressed as that found in the case of diet I. Probably this may account for the fact that here we did not find a significantly higher duodenal flow of bypass N; nor did the ammonia concentration of the rumen decrease significantly.

When diet III was supplemented with avoparcin, the apparent ruminal digestibility of N increased from 15.3% to 22.2% at a lower duodenal content of microbial N. At the same time, the ammonia concentration of the rumen was identical in the two groups.

The results described above indicate that the effect of avoparcin on the parameters of ruminal N metabolism — with the exception of the duodenal flow of microbial N — is not independent of the composition of feed as substrate. This may be one of the explanations for the often conflicting results published in the special literature.

Using avoparcin in a ration of high grain content, Darden et al. (1985) found no change in the ammonia concentration of the ruminal fluid. At the same time, Chalupa et al. (1981) and Froetschel et al. (1983) reported a decrease in ammonia concentration, in agreement with our findings.

In groups I+A and II+A of this study, avoparcin was found to decrease the true digestibility of N. This effect is consistent with that observed for the ionophore antibiotic monensin (Van Nevel and Demeyer, 1977; Russell et al., 1981). Several research teams (Poos et al., 1979; Muntifering et al., 1981) reported that upon the administration of monensin the passage of bacterial N to the duodenum decreased while that of feed N increased. These results are consistent with what was observed in this experiment in group I+A fed avoparcin. The changes observed in ruminal N metabolism after monensin administration are attributed to the inhibition of proteolysis and of the deamination process (Whetstone et al., 1981). The proteolysis-inhibiting effect of avoparcin has not been demonstrated yet. At the same time, the *in vivo* studies of Froetschel et al. (1983) clearly demonstrated that avoparcin inhibits the process of deamination during protein degra-

dation, leading to a reduction in ruminal ammonia content and a simultaneous rise in the concentration of α-amino N. Jouany and Thivend (1986) studied the effect of avoparcin on ruminal N metabolism in an *in vitro* experiment. Avoparcin was found to exert an inhibitory effect on protein digestibility; the higher the ruminal degradability of dietary protein, the stronger that inhibitory effect. Our experimental results support this statement: avoparcin supplementation (I+A, II+A) of rations I and II (RDP content: 74% and 57%, respectively) decreased the true ruminal digestibility of N, while that of diet III of the lowest (48%) RDP content produced no change in the ruminal degradation of N. However, irrespective of dietary RDP content, all three experimental treatments reduced the efficiency of microbial synthesis. This finding is consistent with data reported by the authors mentioned above.

Our experimental results described above indicate that avoparcin has effects comparable to those of ionophore antibiotics both on the processes of ruminal fermentation and the ruminal degradation of protein. As a result of the reduced microbial breakdown of dietary protein (due to the inhibition of deamination) the bypass protein content of the duodenum increases. As the postruminal digestion of dietary protein is more efficient than that of microbial protein (Van Soest, 1982), the apparent digestibility of N in the animal's intestinal tract will probably increase. A better protein or amino acid supply of the host animal may also be facilitated by the direct effects exerted by avoparcin in the intestinal tract. In rats treated with avoparcin, Parker et al. (1984) measured higher dipeptidase activity in the mucosal cells of the intestine. Following avoparcin administration, McGregor and Armstrong (1984) observed higher amino acid absorption from the small intestine of sheep.

The favourable changes produced by avoparcin in the ruminal degradation of nutrients affect both the energy and the protein metabolism of ruminant animals. Thus, the results of our digestive-physiological studies support the beneficial effects of avoparcin observed in the increased body weight gain and feed conversion efficiency of growing animals.

Acknowledgement

This work was supported by a research grant from the National Research Fund of Hungary (OTKA), no. 158/1990.

References

Aguilar, A. A. and Depeters, E. J. (1988): Modification for cannulation of duodenum with flexible, T-shaped cannula in goats. Small Ruminant Res. **1,** 73–79.

Chalupa, W. (1984): Manipulation of rumen fermentation. In: Haresign, W. and Cole, D. J. A. (eds) Recent Advances in Animal Nutrition. Butterworths, London, p. 143–160.

Chalupa, W., Oppegard, C., Williams, H. C., Bloch, B. and Perkins, G. (1981): Effect of avoparcin on rumen environment and fermentation. J. Anim. Sci. **53,** Suppl. 1, 387 (abs. 638).

Chaney, A. L. and Marbach, E. P. (1962): Modified reagents for determination of urea and ammonia. Clin. Chem. **8,** 130.

Christian, R. R. and Coup, M. R. (1954): Measurement of feed intake by grazing cattle and sheep. VI. The determination of chromic oxide in faeces. New Zealand J. Sci. and Tech. Sec. A. **36,** 328–330.

Cottyn, B. G., Fiems, L. O., Boucque, C. V., Aerts, J. V. and Buysse, F. X. (1983): Effect of monensin-sodium and avoparcin on digestibility and rumen fermention. Zeitschr. Tierphysiol. Tierernähr. Futtermittelkde **49,** 277–286.

Crampton, E. W. and Maynard, L. A. (1938): The relation of cellulose and lignin content to the nutritive value of animal feeds. J. Nutr. **15,** 383–395.

Darden, D. E., Merchen, N. R., Berger, L. L. and Fahey, G. C. (1985): Effects of avoparcin, lasalocid and monensin on sites of nutrient digestion in beef steers. Nutr. Rep. Int. **31,** 979–989.

Dehareng, D. and Godeau, J. M. (1989): Polyethylene glycol dilution curves: a comparison of apparent ruminal liquid volumes and dilution rates estimated from either ruminal or duodenal fluid samples of Friesian cows. J. Anim. Physiol. a. Anim. Nutr. **62,** 268–276.

Dyer, I. A., Koes, R. M., Herlugson, M. L., Ojikutu, L. B., Preston, R. L., Zimmer, P. and DeLay, R. (1980): Effect of avoparcin and monensin on performance of finishing heifers. J. Anim. Sci. **51,** 843–846.

Faichney, G. J. (1980): The use of markers to measure digesta flow from the stomach of sheep fed once daily. J. Agric. Sci. **94,** 313–318.

Froetschel, M. A., Croom, W. J., Gaskins, H. R., Leonard, E. S. and Whitacre, M. D. (1983): Effects of avoparcin on ruminal propionate production and amino acid degradation in sheep fed high and low fiber diets. J. Nutr. **113,** 1355–1362.

Günther, K. D. (1987): The effectiveness of the growth promoter Avotan in pig fattening in the framework of liquid feeding with maize cob mix and whey (in German). Schweinewelt **12,** 18–22.

Hecker, J. F. (1974): Experimental Surgery on Small Ruminants. Butterworths, London.

Herold, I., Szabó, P., Kováts, K., Molnár, Z. and Faragó J. (1984): Effect of certain growth promoters on production and FCR of fattening pigs. (in Hungarian) Állattenyésztés és Takarmányozás **33,** 445–452.

Hyden, S. (1955): The recovery of polyethylene glycol after passage through the digestive tract. Kgluntbruks. Hogskil. Ann. **22,** 411–424.

Ingle, D. L., Dalrymple, R. H. and Kiernan, J. A. (1978): Avoparcin, a ruminant growth promoting antibiotic. J. Anim. Sci. **47,** Suppl. 1. 414. (abs. 524).

Johnson, R. J., Herlugson, M. L., Ojikutu, L. B., Cordova, G., Dyer, I. A., Zimmer, P. and DeLay, R. (1979): Effect of avoparcin and monensin on feedlot performance of beef cattle. J. Anim. Sci. **48,** 1338–1342.

Jouany, J. P. and Thivend, P. (1986): *In vitro* effects of avoparcin on protein degradability and rumen fermentation. Anim. Feed. Sci. Techn. **15,** 215–229.

McGregor, R. C. and Armstrong, D. G. (1984): The feed antibiotic avoparcin and net uptake of amino acids from the small intestine of sheep. Can. J. Anim. Sci. **64,** 134–135.

MSz 6830 (Hungarian Standard no. 6830): Determination of the nutritive value of feeds. Chemical tests and calculations (in Hungarian).

Muntifering, R. B., Theurer, B. and Noon, T. H. (1981): Effects of monensin on site and extent of whole corn digestion and bacterial protein synthesis in beef steers. J. Anim. Sci. **53,** 1565–1573.

Ørskov, E. R. and McDonald, I. (1979): The estimate of protein degradability in the rumen from incubation measurements weighed according to the rate of passage. J. Agric. Sci. **92,** 499–503.

Parker, D. S., McGregor, R. C., Finlayson, H. J., Stockill, P. and Balios, J. (1984): The effect of including avoparcin in the diet on cell turnover and enzyme activity in the mucosa of the rat small intestine. Can. J. Anim. Sci. **64,** 136–137.

Poos, M. I., Hanson, T. L. and Klopfenstein, T. J. (1979): Monensin effects on diet digestibility, ruminal protein bypass and microbial protein synthesis. J. Anim. Sci. **48,** 1516–1523.

Roth, F. X. and Kirchgessner, M. (1975): Nutritive effect of the new antibiotic avoparcin-lauryl-sulfate on fattening pigs (in German). Züchtungskunde **47,** 104–111.

Russell, J. B., Bottje, W. G. and Cotta, M. A. (1981): Degradation of protein by mixed cultures of rumen bacteria: identification of *Streptococcus bovis* as an actively proteolytic rumen bacterium. J. Anim. Sci. **53,** 242–252.

Speth, J., Greensten, M. and Maise, W. (1981): The mechanism of action of avoparcin, a glycopeptide antibiotic. Abstracts 81st ASM Meeting. A15.

Stewart, C. S. and Duncan, S. H. (1985): The effect of avoparcin on cellulolytic bacteria of the ovine rumen. J. Gen. Microbiol. **131,** 427–435.

Stokes, S. R., Hoover, W. H., Miller, T. K. and Blauweikel, R. (1991): Ruminal digestion and microbial utilization of diets varying in type of carbohydrate and protein. J. Dairy Sci., **74,** 871–881.

Supelco Inc. (1975): G. C. Separation of VFA C2-C5. Bull. 749 Supelco Inc., Bellefonte, PA.

Sváb, J. (1981): Biometric Methods in Agricultural Research (in Hungarian). Mezőgazdasági Kiadó. Budapest.

Szelényi, M., Jécsai, Gy., Juhász, B. and Fébel, H. (1989): Effect of different growth promoters on the metabolism and fattening performance of young lambs (in Hungarian). Phylaxia Állatorvosi Közlemények **25,** 99–106.

Udén, P., Colucci, P. E. and Van Soest, P. J. (1980): Investigation of chromium, cerium and cobalt as markers in digesta. Rate of passage studies. J. Sci. Food Agric. **31,** 625–632.

Unsworth, E. F., McCullough, I. I., McCullough, T. A., O'Neill, D. G., Steen, R. W. J. and Titterington, C. I. (1985): The effect of avoparcin and monensin on the performance of growing and finishing cattle offered grass silage-based diets. Anim. Prod. **41,** 75–82.

Van Es, A. J. H. and Van der Meer, J. M. (1980): Methods of analysis for predicting the energy and protein value of feeds for farm animals. Workshop on Methodology of Analysis of Feedstuffs for Ruminants, Lelystad, May 27–29.

Van Nevel, C. J. and Demeyer, D. I. (1977): Effect of monensin on rumen metabolism *in vitro*. Appl. Environ. Microbiol. **34,** 251–257.

Van Soest, P. J. (1982): Nutritional Ecology of the Ruminant. O&B Books, Inc., Corvallis Oregon, 230 pp.

Van Soest, P. J. and Robertson, J. B. (1985): Analysis of Forages and Fibrous Foods. AS 613 Manual, Dep. Anim. Sci., Cornell Univ., Ithaca, NY.

Whetstone, H. D., Davis, C. L. and Bryant, M. P. (1981): Effect of monensin on breakdown of protein by ruminal microorganisms *in vitro*. J. Anim. Sci. **53,** 803–809.

Zinn, R. A. and Owens, F. N. (1986): A rapid procedure for purine measurement and its use for estimating net ruminal protein synthesis. Can. J. Anim. Sci. **66,** 157–166.

Acta Veterinaria Hungarica 43 (2–3), pp. 247–257 (1995)

SEASONAL CHANGES IN SPERM PARAMETERS OF BRITISH MILK RAMS

P. SARLÓS and A. MOLNÁR

Research Institute for Animal Breeding and Nutrition, H–2053 Herceghalom, Hungary

(Received November 23, 1994)

Seasonal changes in the sperm parameters of British Milk rams were studied at weekly intervals over a period of 12 months. By comparing the seasonal averages of the test parameters, significant differences were determined in several cases. Substantial differences were found in the averages of quantitative parameters between autumn and the other seasons. As regards the qualitative parameters, the results obtained in summer and autumn were significantly different from those measured in winter and spring. The ratio of deformed spermatozoa was outstandingly high (22.72%) in August. A close negative correlation was established between sperm motility and the incidence of acrosomal defects ($r = -0.52$; $P < 0.1\%$). The length of natural daylight and daily average temperature were found to exert an influence on the sperm parameters. In autumn, the combined effect of the two weather parameters shows a significant correlation with the concentration of spermatozoa ($r = -0.62$; $P < 0.1\%$). As British Milk rams show well-balanced sperm production throughout the year with only minor fluctuations, they can be used for reproduction all the year round.

Key words: Seasonal changes, sperm concentration, motility, sperm anomalies, British Milk ram

In the temperate zone, the reproductive activity of most sheep breeds is known to be linked to season: it takes place in the autumn and is associated with the photoperiod, i.e. the change of the length of natural daylight. While seasonality is more typical of the reproduction of female sheep, it can undoubtedly be observed also in the rams of certain breeds.

The seasonality of reproductive function in rams has been studied in several breeds including Finnish Landrace and Tasmanian Merino (Islam and Land, 1977), Romney (Xu et al., 1993), Suffolk and Hampshire (Cupps et al., 1960), Suffolk, Texel and Dorset Horn (Boland et al., 1985), as well as Suffolk and a hybrid developed from the Dorset, Leicester, and Suffolk breeds (Dufour et al., 1984).

The objective of the experiments presented in this paper was to determine the seasonal fluctuation of sperm parameters in British Milk rams, a breed hitherto not known here, under the climatic conditions typical of Hungary, and to assess how these changes affect the rams' fertilizing capacity.

0236–6290/95/$ 4.00 © 1995 *Akadémiai Kiadó, Budapest*

Materials and methods

The six rams used in the experiment were born in 1989. They arrived at our experimental farm in Herceghalom from Southern Scotland and from the Midlands of England in 1990. The studies were started on 1 December 1991 and completed on 30 November 1992. During the period of experiment, samples were taken and studies were performed once a week, always at the same time.

The rams tupped three times per week on the average. In the autumn season, from 18 September to mid-October the number of services per day was unverifiable because of the harem-like mating system applied. In order to collect semen for examination, the rams were made to mount oestrous ewes. The quantity of the ejaculates was determined, sperm concentration (sperm count per ml) was measured by photometry, and total sperm count per ejaculate was calculated. Fresh samples were examined for sperm motility, and were scored from 1 to 5 ($1 < 5$).

During the morphological evaluation of sperm, the ratio of spermatozoa with abnormalities affecting the fertilizing capacity was determined. For that examination, a sperm smear was prepared from suitably extended fresh semen samples, stained according to the method of Cerovsky (1976), then 100 spermatozoa were examined at 1000-fold magnification for the occurrence of abnormal forms.

The following abnormalities were observed: (1) Acrosome abnormalities: (a) missing apical ridge (MAR); (b) damaged apical ridge (DAR); (c) loose acrosomal cap (LAC); knobbed acrosome; (e) "tilted cap" (acrosome in tilted position. (2) Head abnormalities: (a) multiple head; (b) macro head; (c) pyriform head; (d) round head; (e) oblonged head; (f) micro head; (g) SME defect. (3) Tail abnormalities: (a) hairpin curved sperm tail; (b) single bend tail; (c) coiled tail. (4) Droplets: (a) proximal droplet; (b) distal droplet. (5) Anomalies of the middle piece: (a) double middle piece; (b) retroaxial tail implantation; (c) pseudo Dag defect.

The different acrosome and head abnormalities were combined for data processing. Mathematical evaluation of the data was performed by the t-test and by regression analysis. The different seasons included the following months: winter: December, January, February;, spring: March, April, May; summer: June, July, August; autumn: September, October, November.

Results

The seasonal fluctuations in the average values of the sperm parameters studied are summarized in Table 1.

Table 1

Average values of sperm parameters in ejaculates of British Milk rams in different seasons

	Winter		Spring		Summer		Autumn	
	n = 60		n = 77		n = 60		n = 66	
	x	s	x	s	x	s	x	s
Ejaculate volume (ml)	*1.34	0.45	1.12	0.35	1.22	0.41	1.16	0.56
Concentration of spermatozoa ($\times 10^9$/ml)	3.64	0.70	*4.03	0.80	3.91	0.89	2.42	1.37
Motility (1 < 5)	*4.79	0.60	4.46	0.99	4.45	1.03	4.25	1.26
Acrosomal defects (%)	1.68	2.96	2.17	3.38	*3.27	5.20	2.55	4.24
Proximal droplet (%)	0.18	0.46	*0.34	0.90	0.19	0.61	0.20	1.54
Distal droplet (%)	*0.83	1.80	0.63	1.15	0.58	1.98	0.20	0.46
Single bend tail (%)	0.02	0.12	0.06	0.29	0.14	0.73	*0.21	1.09
Coiled tail (%)	0.95	1.32	*1.53	2.20	1.04	3.71	0.85	3.60
Hairpin curved tail (%)	*4.45	5.38	3.63	4.55	3.20	6.26	1.61	2.64
Retroaxial tail implantation (%)	3.67	5.19	2.94	4.26	1.10	2.81	*4.49	6.37
All anomalies (%)	13.80	7.94	13.74	8.28	*15.70	12.76	13.73	10.97

*maximum value, underscored figures represent minimum values

Ejaculate volume was the largest (1.34 ml) in winter and the smallest (1.12 ml) in spring; in summer it showed an increase, then it decreased again. The maximum average ejaculate volume (1.52 ml) was found in November (Fig. 2). The average concentration of spermatozoa (sperm count per ml) was similar in spring and in summer, and gave the lowest value in the autumn season. Sperm motility scores were the highest in winter, the values found in autumn were lower by a score of 0.5 (Fig. 3).

As regards the average incidence of different sperm abnormalities, acrosomal defects occurred least often in winter (1.68%) and showed the highest average incidence in summer (3.27%). The incidence sperm with hairpin curved tail was the highest in winter; subsequently it gradually decreased and reached the minimum in the breeding season.

Spermatozoa with retroaxial tail implantation occurred in the highest number in autumn and showed the lowest incidence in summer. The average incidence of all sperm anomalies was the highest in summer and the lowest in autumn. The average value found in autumn was similar to those obtained in winter and in spring. The other sperm abnormalities also showed minor seasonal fluctuations. As these latter had a negligible impact on the total incidence of sperm abnormalities, their incidence values were combined (Fig. 1). For all sperm parameters, the average values found in different seasons were compared by the t-test (Table 2). Significant seasonal differences were observed in the incidence rate of sperm with retroaxial tail implantation and with hairpin curved tail.

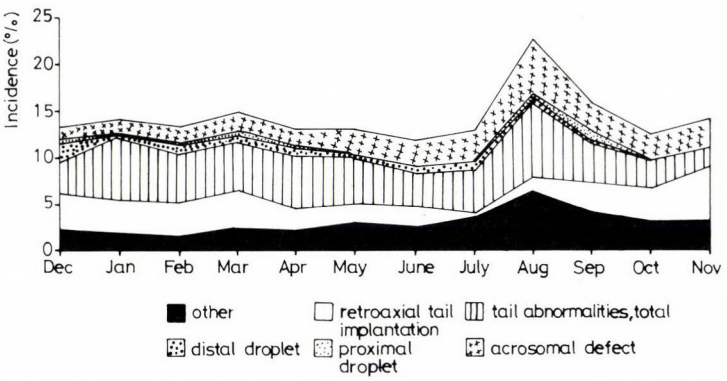


Fig. 1. Monthly average incidences of sperm abnormalities and their ratio within all anomalies

As regards the seasonal incidence of acrosomal defects, a significant difference was demonstrable only between the winter and the summer value. No significant seasonal difference was found in the incidence rate of proximal droplet, single bend tail, coiled tail, and all anomalies.

Table 2

Comparison of averages of sperm parameters in different seasons by the t-test

Parameter	Season	Winter	Spring	Summer	Autumn
Ejaculate volume	Winter		*		
	Spring				
	Summer				
	Autumn				
Concentration of spermatozoa	Winter		*		***
	Spring				***
	Summer				***
	Autumn				
Total number of spermatozoa per ejaculate	Winter				*
	Spring				**
	Summer				**
	Autumn				
Motility	Winter		*	*	
	Spring				
	Summer				
	Autumn				
Acrosomal defects	Winter			*	
	Spring				
	Summer				
	Autumn				
Hairpin curved tail	Winter				***
	Spring				**
	Summer				
	Autumn				
Retroaxial tail implantation	Winter			**	
	Spring			**	
	Summer				***
	Autumn				

* = P < 5%; ** = P < 1%; *** = P < 0.1%

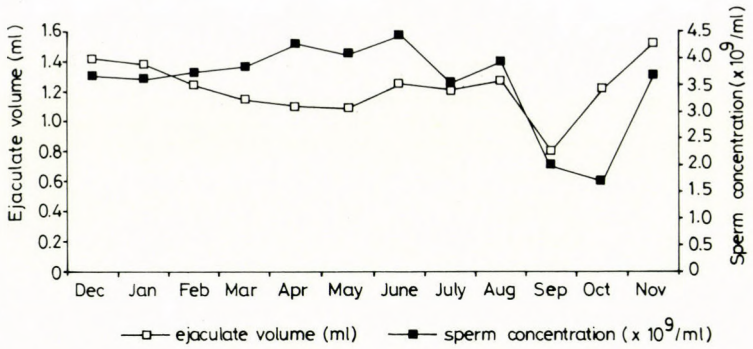


Fig. 2. Monthly average values of ejaculate volume (ml) and sperm concentration ($\times 10^9$/ml) in a 12-month study

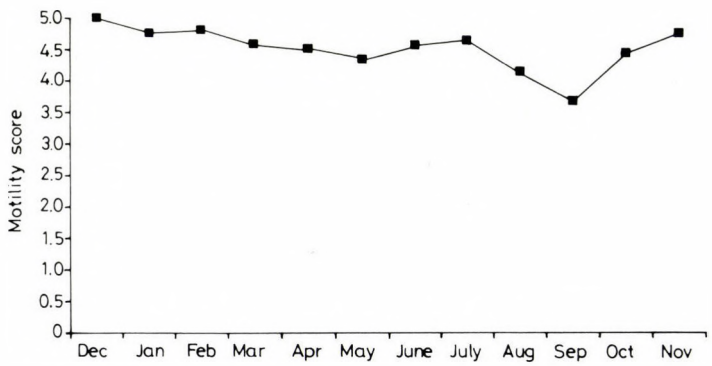


Fig. 3. Monthly averages of sperm motility scores in a 12-month study

Significant seasonal differences were demonstrated in ejaculate volume, sperm concentration, and total sperm count per ejaculate. The average values obtained in autumn significantly differed from those found in winter, spring and summer. Significant differences were observed also between the winter and spring as well as between the winter and summer values of sperm motility.

In order to establish correlations between different parameters, besides the comparison of data measured during the 12 months, correlations between the sperm parameters were determined for every season (Table 3).

Table 3

Correlation values between different sperm parameters

Parameter	Season	Ejaculate volume	Sperm concentration	Motility
Ejaculate volume	Yearly			
	Winter			
	Spring			–0.37**
	Summer			–0.32*
	Autumn			–0.34*
Concentration of spermatozoa	Yearly	0.25**		
	Winter			
	Spring	0.30*		–0.37**
	Summer			
	Autumn	0.37**		
Acrosomal defects	Yearly	0.32***	0.23*	–0.52***
	Winter	0.42**	0.31*	
	Spring	0.32**	0.37**	–0.57***
	Summer	0.40**	0.31*	–0.84***
	Autumn	0.30**		
Hairpin curved tail	Yearly	–0.26**		
	Winter			
	Spring			
	Summer	–0.46**		
	Autumn	–0.36**		
All tail anomalies	Yearly	–0.29**		
	Winter			
	Spring			
	Summer	–0.48***		
	Autumn	–0.29*		
Retroaxial tail implantation	Yearly			
	Winter			
	Spring		0.27*	
	Summer			
	Autumn			
All anomalies	Yearly			
	Winter			
	Spring			
	Summer			–0.43**
	Autumn			

* = P < 5%; ** = P < 1%; *** = P < 0.1%

Significant correlations were demonstrated between ejaculate volume and the incidence of acrosomal defects both on year level ($r = 0.32$; $P < 0.1\%$) and in all seasons. The coefficients of correlation found in winter ($r = 0.42$; $P < 1\%$) and summer ($r = 0.40$; $P < 1\%$) were higher than those obtained in spring ($r = 0.32$; $P < 1\%$) and autumn ($r = 0.30$; $P < 5\%$). Table 3 shows that ejaculate volume was in a significant correlation also with the incidence rates of hairpin curved tail and all tail anomalies, as well as with the concentration and the motility of spermatozoa.

The concentration of spermatozoa was in a low correlation with the incidence of retroaxial tail implantation ($r = 0.27$; $P < 5\%$) and with the yearly incidence of acrosomal defects ($r = 0.23$; $P < 5\%$), and showed a somewhat stronger correlation with the incidence of acrosomal defects in the spring ($r = 0.37$; $P < 1\%$) and a correlation identical with the latter, but of opposite sign, with sperm motility observed in the spring ($r = -0.37$; $P < 1\%$).

In addition to the above correlations, motility was in a close negative correlation with the incidence of acrosomal defects: the correlation found in summer ($r = -0.84$; $P < 0.1\%$) was closer than that obtained on year level ($r = -0.52$; $P < 0.1\%$) and in spring ($r = -0.57$; $P < 0.1\%$). A moderate correlation was established between motility and the incidence of all anomalies. An analysis of the effect exerted by the length of natural daylight revealed that the length of natural daylight was in negative correlation with the incidence of sperm with retroaxial tail implantation ($r = -0.23$; $P < 1\%$).

In addition, the length of natural daylight was correlated with the incidence rate of all anomalies in summer ($r = -0.34$; $P < 0.1\%$) and with the concentration of spermatozoa ($r = -0.51$; $P < 0.1\%$) as well as with sperm motility ($r = -0.36$; $P < 1\%$) in autumn (Table 4).

Daily average temperature was in a significant negative correlation with the yearly incidence rate of sperm with retroaxial tail implantation ($r = -0.25$; $P < 1\%$). It was negatively correlated with the autumn values of the concentration of spermatozoa ($r = -0.61$; $P < 0.1\%$), ejaculate volume ($r = -0.31$; $P < 5\%$) and sperm motility ($r = -0.31$; $P < 5\%$) (Table 4).

The combined effect exerted by daylight in hours and daily average temperature on sperm parameters was also studied. It was found to be correlated with the concentration of spermatozoa ($R = 0.28$; $P < 0.1\%$), with the ratio of sperm with retroaxial tail implantation ($R = 0.26$; $P < 0.1\%$), in the summer season with the incidence of all sperm anomalies ($R = 0.34$; $P < 1\%$), and in autumn with ejaculate volume ($R = 0.34$; $P < 1\%$) and the concentration of spermatozoa ($R = 0.62$; $P < 0.1\%$).

Table 4

Effect of changes of natural daylight and daily average temperature on sperm parameters

Parameter	Season	Natural daylight	Daily average temperature	Daylight (h) + average temperature
Ejaculate volume	Yearly			
	Winter			
	Spring			
	Summer			
	Autumn		–0.31*	0.34**
Concentration of spermatozoa	Yearly			0.28***
	Winter			
	Spring			
	Summer			
	Autumn	–0.51***	–0.61***	0.62***
Motility	Yearly			
	Winter			
	Spring			
	Summer			
	Autumn	0.36**	–0.31*	
Retroaxial tail implantation	Yearly	–0.23*	–0.25*	0.26***
	Winter			
	Spring			
	Summer			
	Autumn			
All anomalies	Yearly			
	Winter			
	Spring			
	Summer	–0.34**		–0.34**
	Autumn			

* = $P < 5\%$; ** = $P < 1\%$; *** = $P < 0.1\%$

Discussion

The results indicate that sperm parameters are subject to seasonal fluctuations. Significant seasonal differences were found in ejaculate volume, in the concentration of spermatozoa, in sperm motility, and in the incidence of some sperm abnormalities.

Taking the monthly average values, ejaculate volume was the highest in November. Sanford et al. (1977) reported that ejaculate volume increased from August (0.72 ml) to November (1.03 ml). In this study, higher values were measured (1.27 and 1.52 ml, respectively). In view of the harem-like mating system used at the experimental farm, the sperm count and ejaculate volume data measured in the autumn season can be accepted only with some reserve because of the rams' excessive sexual utilization.

With the exception of August, the qualitative parameters of sperm show minor fluctuations over the year. According to Colas and Courot (1977), Ile de France rams have a 10.3% incidence of abnormal sperm in the autumn.

In this study, the incidence of all sperm anomalies in autumn (13.73%) and in spring (13.74%) was not significantly different. The highest incidence of abnormal spermatozoa (22.77%) was observed in August, when the daily average temperature was 26.5 °C, several degrees higher than the average of many years. The outstandingly high incidence of abnormal spermatozoa must have been due to that high temperature. Cupps et al. (1960) reported similar findings (a 26% incidence of abnormal spermatozoa) in the Suffolk and Hampshire breeds.

Sperm motility also shows seasonal fluctuations: the highest values were found in winter and poorer sperm motility was observed in autumn. In Suffolk and DLS rams, Dufour et al. (1984) found poorer sperm motility in the spring season.

According to the results of this study, certain sperm parameters correlate with each other. Most often it is the ejaculate volume which correlates with other parameters. The incidence of spermatozoa affected with acrosomal defects and tail abnormalities rises with the increase in ejaculate volume and sperm concentration. Acrosomal defects markedly impair the motility of spermatozoa. Interestingly, tail abnormalities were not found to exert such an effect. In addition to acrosomal defects, the incidence of all anomalies was the only qualitative sperm parameter that adversely affected sperm motility.

In contrast to the results obtained by Boland et al. (1985) for other breeds, in this study the daily average temperature and the length of natural daylight were found to influence the sperm parameters. Both weather parameters exerted a slight effect on the incidence of abnormal spermatozoa. Only with the concentration of spermatozoa did they have a stronger correlation. The same finding was obtained when the combined effect of the two weather parameters was studied.

The objective of this study was to determine the seasonal fluctuation of the sperm parameters in British Milk rams and the influence it exerted on the rams' fertilizing capacity. According to the findings, the semen production and parameters of this breed are rather consistent and show only minor fluctuations throughout the year; thus, under the climatic conditions typical of Hungary British Milk rams can be used for reproduction also outside the breeding season.

References

Boland, M. P., Alkamali, A. A., Crosby, T. F., Haynes, N. B., Howles, C. M., Kelleher, P. L. and Gordon, I. (1985): The influence of breed, season and photoperiod on semen characteristics, testicular size, libido and plasma hormone concentration in rams. Anim. Reprod. Sci. **9,** 241–252.

Cerovsky, J. (1976): Metoda barvemi kancich spermii pro morfologické hodnoceni. Zivocisna Vyroba **21,** 361.

Colas, G. and Courot, M. (1977): Production of spermatozoa, storage of semen and artifical insemination in the sheep. Management of reproduction in sheep and goats, Symposium, Madison, Wisconsin. **24,** 31.

Cupps, P. T., McGowean, B., Rahlman, D. F., Reddon, A. R. and Weir, W. C. (1960): Seasonal changes in the semen of rams. J. Anim. Sci. **19,** 208.

Dufour, J. J., Fahmy, M. H. and Minvielle, F. (1984): Seasonal changes in breeding activity, testicular size, testosterone concentration and seminal characteristics in rams with long or short breeding season. J. Anim. Sci. **58,** 416–422.

Islam, A. B. M. M. and Land, R. B. (1977): Seasonal variation on testis diameter and sperm output of rams of breeds of different prolificacy. Anim. Prod. **25,** 311–317.

Sanford, L. M., Palmer, W. M. and Howland, B. E. (1977): Changes in the profiles of serum LH, FSH and testosterone, and in mating performance and ejaculate volume in the ram during the ovine breeding season. J. Anim. Sci. **45,** 1382.

Xu, Z. Z., McDonald, M. F., McCutcheon, S. N. and Blair, H. T. (1993): Testis size, gonadotrophin secretion and pituitary responsiveness to GnRH in Romney rams during the transition from the non-breeding to the breeding season. Anim. Reprod. Sci. **31,** 99–111.

Acta Veterinaria Hungarica 43 (2–3), pp. 259–267 (1995)

OCCURRENCE OF *FUSARIUM* SPECIES AND ZEARALENONE IN DAIRY CATTLE FEEDS IN VOJVODINA

M. ŠKRINJAR[1], R. D. STUBBLEFIELD[2], E. STOJANOVIĆ[3] and G. DIMIĆ[1]

[1]University of Novi Sad, Faculty of Technology, 21000 Novi Sad, Yugoslavia; [2]USDA, ARS, National Center for Agricultural Utilization Research, IL 61604–3999, U.S.A.; [3]Dairy Plant "Odžačanka", 25250 Odžaci, Yugoslavia

(Received January 30, 1995)

The occurrence of *Fusarium* spp. and zearalenone (F-2 toxin) in dairy cattle feeds was studied during a period of three years (in all seasons) in Vojvodina. *Fusarium* species were found to be very common in feeds. They were classified into 11 different species (*F. chlamydosporum, F. equiseti, F. flocciferum, F. graminearum, F. lateritium, F. moniliforme, F. oxysporum, F. semitectum, F. solani, F. tabacinum* and *F. tricinctum*). Some of these species are known to produce mycotoxins. The highest distribution of *Fusarium* spp. was observed in the autumn and spring of the second research year, when 53 and 56% of the feed samples, respectively, were contaminated with them. *F. oxysporum* and *F. moniliforme* were the most prevalent species throughout the three-year study. Zearalenone was found in various feed samples at concentrations between 140.00 and 960.00 μg/kg in all seasons, except in the summer of the first year, in the winter of the second year and in the autumn of the third research year. Pelleted sugar beet pulp and pelleted malt spent grains were the feeds most contaminated with this toxin. Thirty-five out of the 275 feed samples contained zearalenone and 11 of them were contaminated with zearalenone-producing moulds and zearalenone at the same time.

Key words: *Fusarium* species, zearalenone, dairy cattle feeds, prevalence

Zearalenone or F-2 toxin is an oestrogenic metabolite of several species of the genus *Fusarium* . It was first isolated and partially characterized in 1962 from corn (Stob et al., 1962). At the same time *Gibberella zeae* was isolated from this corn. Culture material of *G. zeae* caused vulvar and mammary enlargement in immature pigs and also induced uterine hypertrophy in ovariectomized mice. Marasas et al. (1984) later identified that strain as *F. graminearum.*

Several authors reported that zearalenone and its metabolites can be transmitted into ruminant and porcine milk. Hagler et al. (1980) investigated transmission of zearalenone into ruminant milk and fed a dairy cow with mixed feed (3 l) containing 5 g of crystalline zearalenone. The cow consumed all the feed and toxin within 10 min. Extracts of milk analyzed by reverse-phase HPLC revealed traces of zearalenone and β-zearalenol in some extracts of cow's milk.

0236–6290/95/$ 4.00 © 1995 *Akadémiai Kiadó, Budapest*

Mirocha et al. (1981) reported that when cows were fed a diet containing a high concentration of zearalenone (25 mg/kg of toxin over 7 days), maximum levels of 480 μg/kg of zearalenone, 508 μg/kg of α-zearalenol and 370 μg/kg of β-zearalenol were found in milk.

Fusarium species are frequently found in cereal grains which act as an excellent substrate for their growth and toxic metabolite production. They are especially widely distributed under the climatic conditions of the temperate zone. Kordi ć et al. (1986) studied the contamination of feeds samples taken from big agricultural units, mash feed factories, livestock farms and individual farms. They found that *Fusarium* species were the commonest moulds in cereals. Zearalenone was found in 38% of the test samples at concentrations between 0.2 and 10 mg/kg.

In the past few years, investigations have been conducted on the prevalence of moulds and mycotoxins (aflatoxins, ochratoxins, zearalenone, trichothecenes) in feeds in Yugoslavia. Some of the results obtained have already been published (Škrinjar et al., 1992*a*, 1992*b*).

This work reports results on the prevalence of *Fusarium* species and zearalenone in dairy cattle feeds.

Materials and methods

Feeds. The feeds originated from four large state farms The studies comprised a period of three years (all seasons).

Mycological analysis. Isolation of moulds from feed samples was performed by the dilution plate technique. Inoculated Petri dishes (in triplicates) with Sabouraud dextrose agar (SDA) and streptomycin (0.01–0.02 %) were incubated for 7 days at 25 °C. After incubation, colonies of the genus *Fusarium* were inoculated on potato dextrose agar (PDA). Identification of *Fusarium* species was performed according to Nelson et al. (1983).

Zearalenone analysis. All feed samples were analyzed for the presence of zearalenone. Qualitative and quantitative determination of this toxin was carried out using a TLC method according to the Official Methods of Analysis of the A.O.A.C. (1990).

Results

A relative high prevalence of mould contamination in dairy cattle feeds caused by the genus *Fusarium* was observed during the three-year study. These moulds were classified into 11 species and belonged to 9 different sections of the genus according to Nelson et al. (1983) (Table 1). Six species (*F. chlamydosporum, F. semitectum, F. equiseti, F. graminearum, F. moniliforme* and

F. oxysporum) are known as zearalenone-producing moulds, two species (*F. tabacinum* and *F. flocciferum*) are zearalenone-negative and for the remaining three species (*F. lateritium, F. tricinctum* and *F. solani*) no unambiguous data are available as regards the ability of zearalenone production (Marasas et al., 1984).

Table 1

Fusarium species isolated from dairy cattle feeds

Section	Species
Eupionnotes	*F. tabacinum* (Beyma) W. Gams
Sporotrichiella	*F. chlamydosporum* Wollenw. & Reinking
	F. tricinctum (Corda) Sacc.
Arthrosporiella	*F. semitectum* Berk. & Rav.
Gibbosum	*F. equiseti* (Corda) Sacc.
Discolor	*F. flocciferum* Corda
	F. graminearum Schwabe
Lateritium	*F. lateritium* Nees
Liseola	*F. moniliforme* Sheldon
Elegans	*F. oxysporum* Schlecht. emend. Snyd. & Hans.
Martiella-Ventricosum	*F. solani* (Mart.) Appel & Wollenw. emend. Snyd. & Hans.

Figures 1, 2 and 3 present the ratios of *Fusarium* species in mycopopulations isolated from the feeds during the three-year period. Figure 1 shows that 36 and 43% of the moulds isolated in summer and winter in the first research year, respectively, belonged to the genus *Fusarium*. During the second year an even higher incidence of *Fusarium* spp. was observed in the autumn (53%) and spring (56%) (Fig. 2). The highest incidence of *Fusarium* contamination during the third research year was found in the summer (43%) and autumn (44%) (Fig. 3).

Among *Fusarium* species, *F. graminearum* is the most important producer of zearalenone. Fortunately, it showed a low distribution in feed samples during this study. This fungus was isolated only from corn silage in the spring of the first research year (Fig. 1).

Fusarium equiseti and *F. chlamydosporum* were isolated from cattle feed samples only in the summer and autumn of the third year, respectively, (Fig. 3). *Fusarium semitectum* was found to be a more common contaminant, occurring in feeds during the first (summer, winter), second (autumn) and third year (autumn, winter) (Figs 1, 2 and 3).

The most common *Fusarium* species during the first and third year was *F. oxysporum* (Figs 1 and 3). This species is known as a soil-based fungus (Mills, 1989). Some populations are very pathogenic while others are vigorous saprophytic colonizers of senescent or damaged plant tissue. Besides zearalenone, some

strains of *F. oxysporum* can produce diacetylnivalenol, moniliformin, T-2 toxin and other toxic metabolites (Marasas et al., 1984).

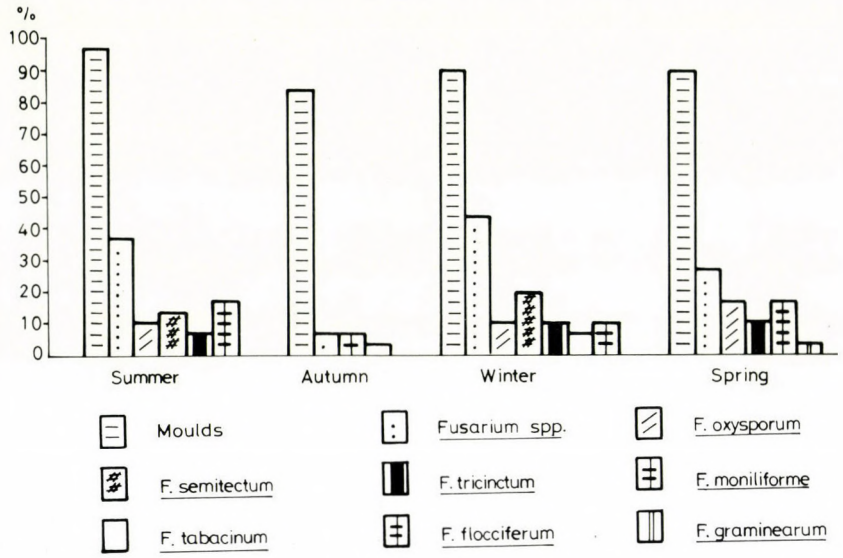


Fig. 1. Contamination of dairy cattle feeds by *Fusarium* species in the first year of study

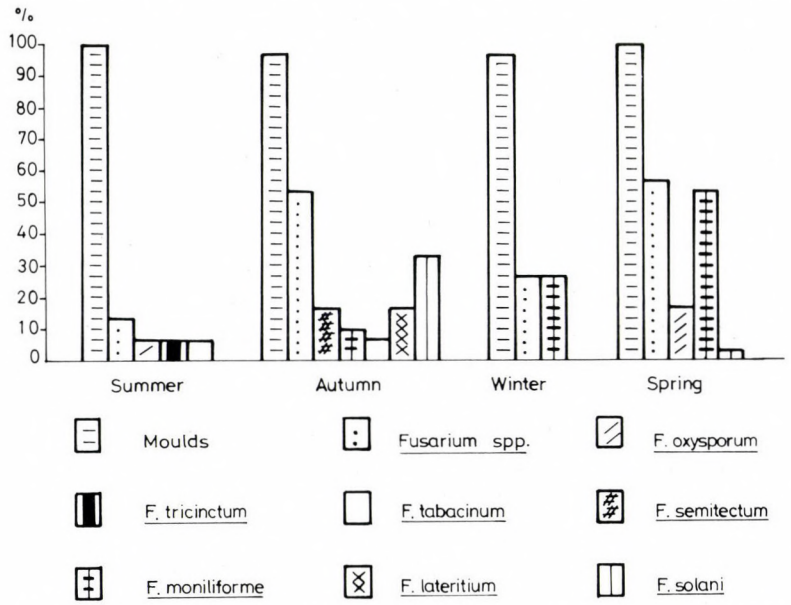


Fig. 2. Contamination of dairy cattle feeds by *Fusarium* species in the second year of study

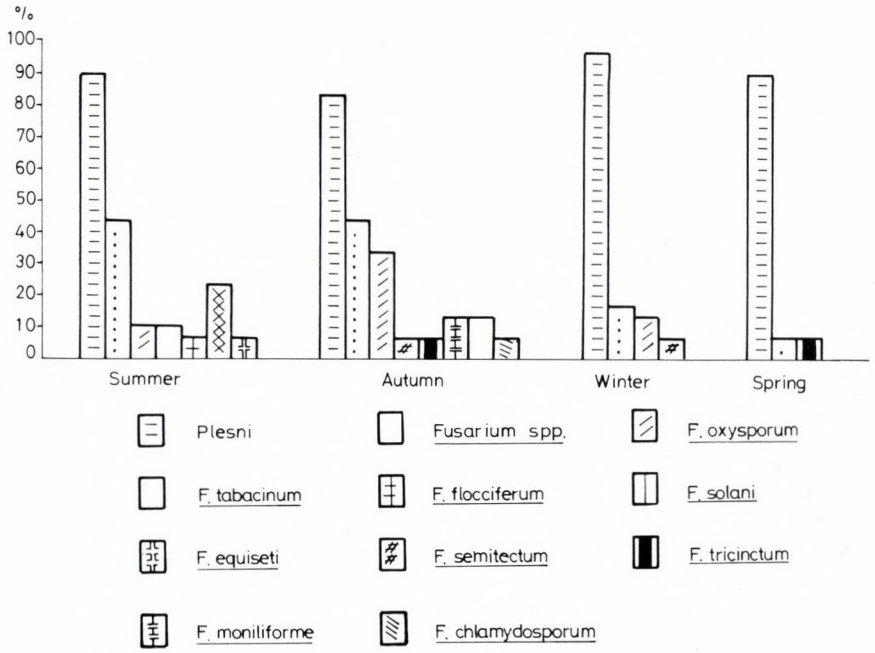


Fig. 3. Contamination of dairy cattle feeds by *Fusarium* species in the third year of study

During the second and third research year (Figs 2 and 3), a high incidence of *F. moniliforme* was observed. It was found that about 52% of moulds isolated in the spring of the second year belonged to the species *F. moniliforme*. This mould is one of the most prevalent seed-borne fungi in corn, sorghum and rice in many countries (Hesseltine and Bothast, 1976; Marasas et al., 1979; Neish et al., 1983). *F. moniliforme* also produces various toxic metabolites such as F-2 toxin, T-2 toxin, deoxynivalenol, diacetoxyscirpenol, gibberellins and moniliformin (Marasas et al., 1984).

In this work, *F. oxysporum* and *F. moniliforme* were mostly isolated from concentrate and dried alfalfa.

Toxigenic *Fusarium* species, isolated in this work, can cause different undesirable effects in animals including cattle. According to Kalra et al. (1973) and Bhat et al. (1978), the consumption of rice straw colonized by *F. equiseti* caused in cattle and buffaloes in India and Pakistan Degnala disease, characterized by oedematous swelling of the legs and necrosis, gangrene and sloughing of the exremities. From China, Qin et al. (1981) reported a similar disease, which affects cattle consuming rice straw contaminated by *F. equiseti* and *F. semitectum*. This bovine disease known as "sore foot disease" is characterized by necrosis of the feet and ears.

Zearalenone, which is a toxic metabolite of several *Fusarium* species, causes oestrogenic syndrome in animals. Some of these species were isolated during this work as mentioned above.

This disease was observed especially in pigs then in dogs, poultry, primates, rodents and sheep, but also in cattle (Mirocha and Christensen, 1974; Hidy et al., 1977; Christensen, 1979; Ueno and Tashiro, 1981). This syndrome involves primarily the genital system. The characteristic clinical signs are hyperaemia and oedematous swelling of the vulva in prepuberal female animals, as well as an enlargement of the mammary glands and hypertrophy of the nipples. Pathological changes in the genital tract are characterized by interstitial oedema and cellular proliferation and metaplasia of the mucosal epithelium of the vagina and cervix. The uterine horns are also enlarged and ovaries are hypoplastic with numerous small follicles. Other reproductive problems such as infertility, mummification, fetal resorptoion, abortions, reduced litter size etc. also occur. In males, hypertrophy of the mammary glands, atrophy of the testes, swelling of the prepuce and decreased libido have also been reported (Mirocha and Christensen, 1974; Kurtz and Mirocha, 1978).

According to Christensen (1979), clinical signs of hyperoestrogenism including swollen and hyperaemic external genitalia have been induced experimentally in cows with pure zearalenone.

The results shown in Table 2 indicate that zearalenone was present in feed samples during the whole research period, except, in summer of the first year, in winter of the second and in autumn of the third year. Concentrations of the toxin ranged between 140.00 and 960.00 μg/kg.

It should be pointed out that pelleted malt spent grains and pelleted sugar beet pulp were the feeds most contaminated with zearalenone (Table 3). The highest concentration of this toxin (960.00 μg/kg) was found in dried alfalfa and pelleted sugar beet pulp.

Thirty-five out of 275 feed samples contained zearalenone. Only 11 of them were contaminated with zearalenone-producing moulds and zearalenone at the same time (Table 2).

Several studies have reported the presence of *Fusarium* species and zearalenone in feeds and cereal grains in Yugoslavia. Šutić et al. (1989) investigated the occurrence of zearalenone in dairy cattle feeds in 1987-1988 and found that 27% of the samples were contaminated with this toxin at concentrations from traces to 30.00 μg/kg. Oberan (1989) reported that 15 out of 16 stored corn grain samples (94%) were positive for zearalenone biosynthesized in nature. Concentrations ranged from 375.00 to 12,500.00 μg/kg. In those samples *F. oxysporum* was found to be the dominant fungus.

Table 2

Number of feed samples contaminated with zearalenone-producing moulds and zearalenone

Research period	No. of feed samples		No. of feed samples contaminated with moulds	No. of feed samples contaminated with *Fusarium* spp.	No. of feed samples contaminated with F-2 toxin/F-2 toxin producing moulds	Concentrations of F-2 toxin, $\mu g \times kg^{-1}$
First research year	S	33	31	12	0/0	–[a]
	A	26	22	2	5/1	140.00 – 165.00
	W	30	27	13	5/1	145.00 – 160.00
	Sp	27	24	7	1/0	960.00
Second research year	S	16	16	2	4/2	220.00 – 240.00
	A	19	18	10	2/2	140.00 – 150.00
	W	19	18	5	0/0	–
	Sp	23	23	13	5/4	240.00 – 480.00
Third research year	S	21	19	9	3/1	240.00
	A	18	15	8	0/0	–
	W	24	23	4	3/0	480.00 – 960.00
	Sp	19	17	1	7/0	220.00 – 480.00

S – Summer; A – Autumn; W – Winter; Sp – Spring; [a] not detected

Table 3

Types of feeds contaminated with zearalenone

Feed	No. of feed samples	No. of feed samples contaminated with F-2 toxin	Percentage of samples contaminated with F-2 toxin
Fresh alfalfa	6	1	17
Dried alfalfa	45	3	7
Hay	20	3	15
Corn silage	60	4	7
Concentrate	57	10	17
Pelleted malt spent grains	12	4	33
Pelleted sugar beet pulp	36	9	25

Acknowledgements

This study was supported by USDA and the Community for Research Work of Vojvodina, through funds made available to the U.S.-Yugoslav Joint Board on Scientific and Technological Cooperation.

References

Bhat, R. V., Nagarajan, V. and Tulpule, P. G. (1978): Health hazards of mycotoxins in India. National Institute of Nutrition, Hyderabad, Indian Council of Medical Research, New Delhi. U.S. Dept. of Commerce, National Technical Information Service, Springfield, Virginia. PB82-170820, 58 p.

Christensen, C. M. (1979): Zearalenone. U.S. Dept. of Commerce, National Technical Information Service, Springfield, Virginia. PB-300, Report no. FDA/BVM-79/139.

Hagler, W. M., Dankó, Gy., Horváth, L., Palyusik, M. and Mirocha, C. J. (1980): Transmission of zearalenone and its metabolite into ruminant milk. Acta Vet. Acad. Sci. Hung. **28,** 209.

Hesseltine, C. W. and Bothast, R. J. (1976): Mold development in ears of corn from tasseling to harvest. Mycologia **69,** 328.

Hidy, P. H., Baldwin, R. S., Greasham, R. L., Keith, C. L. and McMullen, J. R. (1977): Zearalenone and some derivats: production and biological activities. Adv. Appl. Microbiol. **22,** 59.

Kalra, D. S., Bhatia, K. C., Ganatm, O. P. and Chauhan, H. V. S. (1973): An obscure disease (possibly Degnala disease) in buffaloes and cattle. Studies on its epizootiology, pathology and etiology. Haryana Agric. Univ. J. Res. **2,** 256.

Kordić, B., Panin, M., Kandić, S., Lončarević, A. and Muntanola-Cvetković, M. (1986): Results of a three-year microbiological and mycotoxicological examination of feeds in SR Serbia. Special Publication on Second Symposium on Mycotoxins, Academy of Sciences and Arts of Bosnia and Herzegovina, Sarajevo, Vol. **LXXX,** 17.

Kurtz, H. J. and Mirocha, C. J. (1978): Zearalenone (F-2 toxin) induced estrogenic syndrome in swine. In: (Wyllie, T. D. and Morehouse, L. G. (eds) Mycotoxic Fungi, Mycotoxins, Mycotoxicoses. An Encyclopedic Handbook Vol. 2. Marcel Dekker, New York. pp. 256–268.

Marasas, W. F. O., Kriek, N. P. J., Wiggins, V. M., Steyn, P. S., Towers, D. K. and Hastie, T. J. (1979): Incidence, geographic distribution, and toxigenicity of *Fusarium* species in South African corn. Phytopathology **69,** 1181.

Marasas, W. F. O., Nelson, P. E. and Toussoun, T. A. (1984): Toxigenic *Fusarium* species. The Pennsylvania State University Press, University Park and London, xix + 328 pp.

Mills, J. T. (1989): Ecology of mycotoxigenic *Fusarium* species on cereal seeds. J. Food Protection **52,** 737.

Mirocha, C. J. and Cristensen, C. M. (1974): Oestrogenic mycotoxins synthesized by *Fusarium.* In: Purchase, I. F. H. (ed.) Mycotoxins. Elsevier, Amsterdam, pp. 129–148.

Mirocha, C. J., Pathre, S. V. and Robinson, T. S. (1981): Comparative metabolism of zearalenone and transmission into bovine milk. Food and Cosmetic Toxicology **19,** 25.

Neish, G. A., Farnworth, E. R., Greenhalgh, R. and Young J. C. (1983): Observations on the occurrence of *Fusarium* species and their toxins in corn in eastern Ontario. Can. J. Plant Pathol. **5,** 11.

Nelson, P. E., Toussoun, T. A. and Marasas, W. F. O. (1983): *Fusarium* species. An Illustrated Manual for Identification. The Pennsylvania State University Press, University Park and London, ix + 193 pp.

Oberan, Lj. (1989): Biosynthesis of zearalenone (F-2 toxin) in the nature and mycopopulations of investigated corn grains. Special Publication of Third Symposium on Mycotoxins, Academy of Sciences and Arts of Bosnia and Herzegovina, Sarajevo, Vol. **LXXXIX,** 79.

Official Methods of Analysis of the Association of Official Analytical Chemists (1990): Natural Poisons. Scott, P. M. (ed.), pp. 1184–1213.

Qin, G., Zhang, B. S., Wang, Z. Fu and Wang, R. (1981): Studies on the "sore foot disease" of cattle. Acta Vet. Zootech. Sin. **12,** 137.

Stob, J., Baldwin, R. S., Tuite, J., Andrews, F. N. and Gillete, K. G. (1962): Isolation of an anabolic, uterotrophic compound from corn infected with *Gibberella zeae.* Nature **196,** 1318.

Škrinjar, M., Stubblefield, R. D. and Vujičić, I. F. (1992*a*): Ochratoxigenic moulds and ochratoxin A in forages and grain feeds. Acta Vet. Hung. **40,** 185.

Škrinjar, M., Stubblefield, R. D., Vujičić, I. F. and Stojanović, E. (1992*b*): Distribution of aflatoxin-producing moulds and aflatoxins in dairy cattle feed and raw milk. Acta Microbiol. Hung. **39,** 175.

Šutić, M., Svilar, N., Ivanović, D., Tosman, N. and Oljačić, E. (1989): Moulds and mycotoxins in cattle feed. Special Publication of Third Symposium on Mycotoxins, Academy of Sciences and Arts of Bosnia and Herzegovina, Sarajevo, Vol. **LXXXIX,** 109.

Ueno, Y. and Tashiro, F. (1981): α-Zearalenol, a major hepatic metabolite in rats of zearalenone, an estrogenic mycotoxin of *Fusarium* species. J. Biochem. **89,** 563.

Acta Veterinaria Hungarica 43 (2–3), pp. 269–275 (1995)

REDESCRIPTION OF *GOUSSIA NEGLECTA* N. COMB. (NÖLLER, 1920) (APICOMPLEXA; COCCIDIA) AND NOTES ON ITS OCCURRENCE IN THE GUT OF TADPOLES

K. MOLNÁR

Veterinary Medical Research Institute, Hungarian Academy of Sciences,
H–1581 Budapest, P.O. Box 18, Hungary

(Received December 20, 1994)

Coccidian infection in *Rana ridibunda* and *R. esculenta* tadpoles was recorded in Hungarian fish farms. Oocysts enclosed in yellow bodies were found in the faeces and in the intestinal epithelium. Developmental stages also had intraepithelial sites. The species was identified with *Eimeria neglecta* Nöller, 1920; however, on the basis of its oocyst morphology it was transferred to the genus *Goussia*. Besides stages of *Goussia neglecta* n. comb., intranuclear trophozoites of another coccidian species were also recorded.

Key words: *Goussia*-type coccidiosis, tadpoles, anurans, intranuclear stages

Since Grassi first recorded eimeriid coccidia from anurans in 1882, many additional species have been described (reviewed by Upton and McAllister, 1988). Most of the species described thus far have been recovered from adult frogs (Pachinger, 1886; Labbé, 1894; Dobell, 1909; Chen and Desser, 1989). These species belong to the genera *Eimeria* and *Isospora* and inhabit the intestine of frogs. Their oocysts and sporocysts are thick-walled, and the sporocysts have a Stieda-body. Only one species, *Eimeria neglecta*, has been described from tadpoles (Nöller, 1920).

During a study on the coccidia of pond-reared common carp fry, the intestines of tadpoles of *Rana ridibunda* and *R. esculenta* from the same ponds were examined. Many of these tadpoles were heavily infected with a species of *Goussia* identical with the parasite described by Nöller as *Eimeria neglecta*. In this study, the intraepithelial stages of the parasite are described and illustrated, and a redescription of its oocysts is provided.

Materials and methods

Tadpoles of *Rana ridibunda* and *R. esculenta* were seined in Hungarian fish ponds at Százhalombatta (near Budapest) in three consecutive years

0236–6290/95/$ 4.00 © 1995 *Akadémiai Kiadó, Budapest*

(1990–1992). A total of 38 specimens (28 *R. ridibunda* and 10 *R. esculenta*) of different developmental stage were dissected and examined for coccidian infection. The gut of the tadpoles was opened and samples of its content and scrapings from the epithelial mucosa were examined in fresh state by light microscopy. Oocysts, obtained from mucus on the surface of the gut epithelium, were measured and drawn with the aid of a camera lucida. Measurements were made of 50 sporulated oocysts derived from five *Rana ridibunda* tadpoles. All measurements in descriptions are given in micrometers.

Portions of the intestine from some heavily infected tadpoles were fixed in Bouin's solution for 4 h and processed for light microscopy. Four μm thick sections were stained with haematoxylin-eosin or by Farkas-Mallory stain. Photographs were taken using a Zeiss-Jenawal photomicroscope.

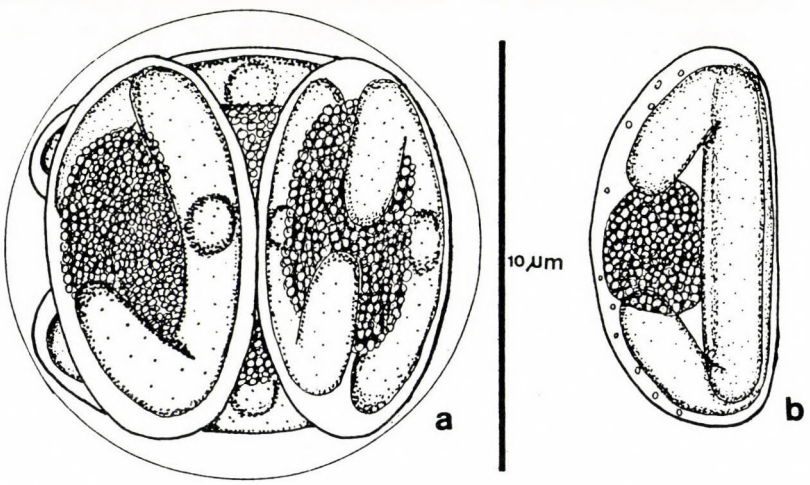


Fig. 1. Schematic illustration of *Goussia neglecta*. (a) Sporulated young oocyst with large elliptical residual bodies in the sporocysts. (b) Old sporocyst with round residual body in lateral view

Results

Oocysts were found in 19 *Rana ridibunda* and 7 *R. esculenta*. The highest prevalence of infection was observed during July in tadpoles that had developed their first pair legs. The prevalence was lower in specimens with two pairs of legs, and lowest in those with no legs.

Oocysts were usually found in the mucus and faeces, and were also seen occasionally in groups of two or three enclosed in yellow bodies (Fig. 2). The oocysts were sporulated and possessed thin oocyst and sporocyst walls. Under a

coverslip the thin wall of the oocysts broke easily and in most cases only free sporocysts were found in the mucus (Fig. 3). Some tadpoles exibited meronts and merozoites but no oocysts.

Goussia neglecta n. comb. (Nöller, 1920)

Host: Tadpoles of *Rana ridibunda*
Additional host: Tadpoles of *Rana esculenta*
Site of infection: Intestinal epithelium
Locality: Fish ponds at Százhalombatta, Hungary
Description (see Figs 1–3)

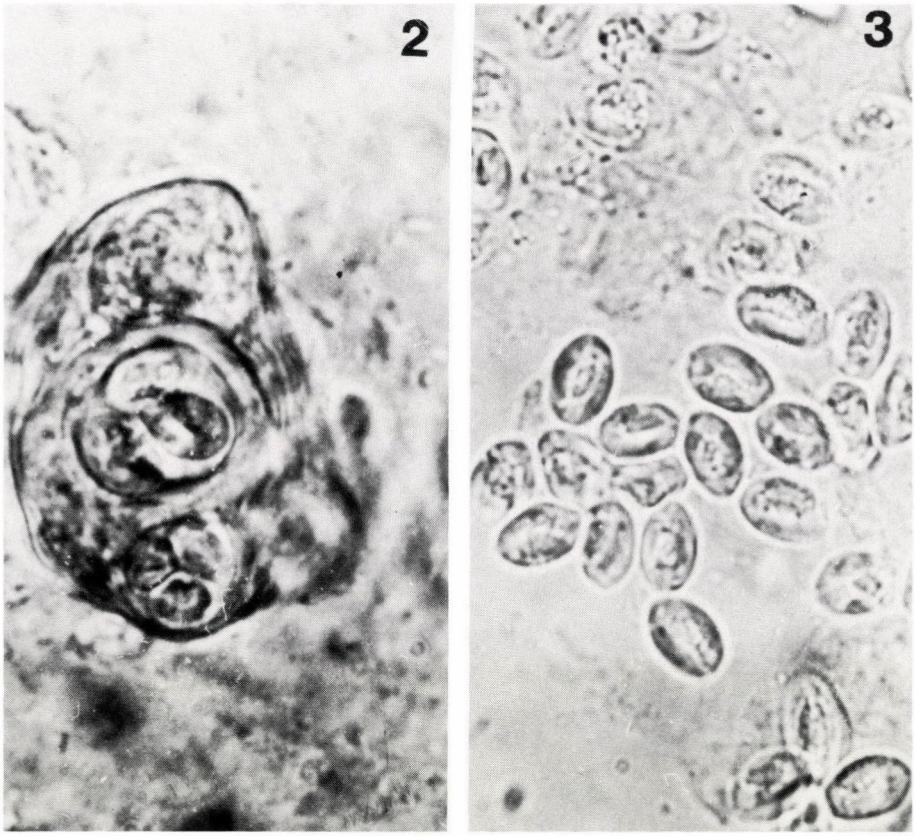


Fig. 2. Sporulated oocysts of *Goussia neglecta* enclosed in a yellow body. Fresh preparation, × 2,000

Fig. 3. Sporocysts of *Goussia neglecta* mechanically excysted from the thin-walled oocysts. Each sporocyst contains two vermiform sporozoites and a granulated ellipsoidal residual body. Fresh preparation, × 1,200

Oocysts (Fig. 1) spherical with a diameter of 10.6 (8.5–12.5). Oocyst wall thin, smooth and colourless; consisting of two halves joined by a suture. Oocysts contain four tightly packed sporocysts; ellipsoidal with one side flattened in lateral view. Sporocysts thin-walled, 8.8 (6.5–10.2) long by 4.8 (4.0–5.7) wide. Each sporocyst contains two vermiform sporozoites with one end reflexed. Sporozoites arranged "head to tail". Length of sporozoites (minus reflexed portion) 8.2 (6.4–9.9) long by 1.8 (1.7-2.0) wide. Sporocyst residue granular and ellipsoidal in young oocysts, measuring 5.0–7.5 × 3.2–3.6. In older oocysts, residue more compact and spherical, measuring 1.7–2.0 in diameter.

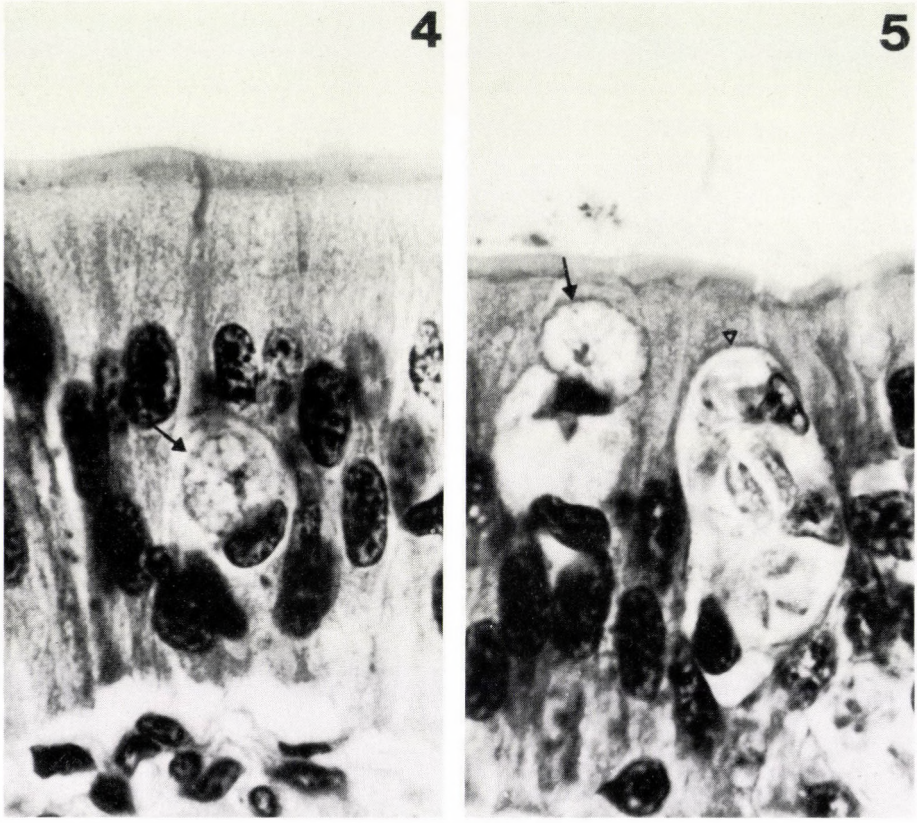


Fig. 4. Goussia neglecta macrogamont (arrow) in the apical cytoplasm of an enterocyte. Haematoxylin and eosin, × 1,200

Fig. 5. Sporocysts of at least three oocysts within the future yellow body (arrow head) and a macrogamont (arrow) in the intestinal epithelium of *Rana ridibunda*. Haematoxylin and eosin, × 1,200

Histological sections from heavily infected tadpoles contained both developmental stages and oocysts. The earliest stages were palely stained meronts containing 8–16 merozoites arranged in parallel. These, as well as gamonts (Figs 4 and 6) and immature oocysts, were observed both in the apical and basal cytoplasm of intestinal epithelial cells. Intranuclear coccidian parasites were also seen occasionally (Fig. 5). The pale staining of intracellular stages made studying of meronts and microgamonts almost impossible, and macrogamonts showed also a relatively pale colour among host cells.

Sporulated oocysts (Fig. 6) were located among epithelial cells or in the subepithelium beneath uninfected cells. The oocysts stained weakly with haematoxylin and eosin, but assumed an intense yellow color with Farkas-Mallory staining.

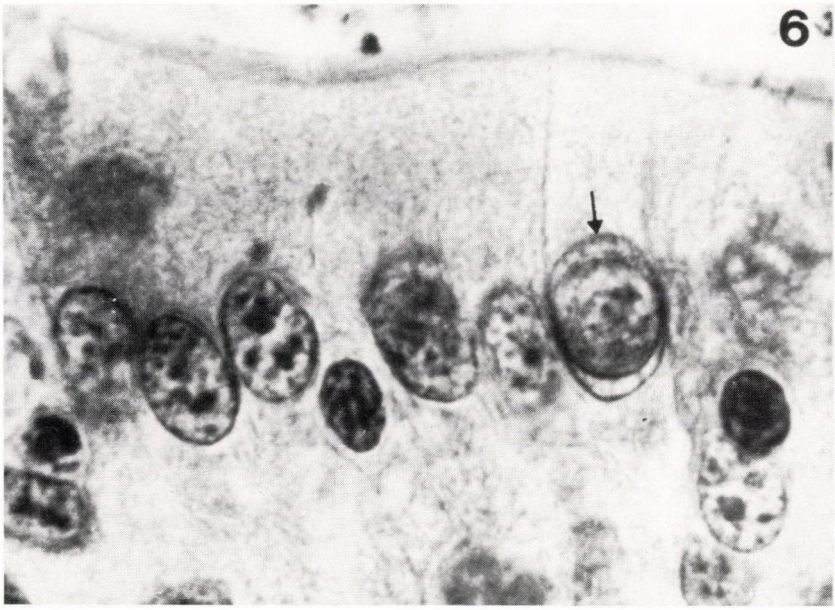


Fig. 6. Intranuclear trophozoites of a coccidium species in the intestinal epithelium. Farkas-Mallory staining, × 2,000

Oocysts found in tadpoles were of typical *Goussia* type; moreover, they resembled *Goussia carpelli* (Léger et Stankovitch, 1921), the causative agent of diffuse coccidiosis of the common carp. There were, however, significant differences between the two species in oocyst structure. The oocysts from tadpoles were less compact and sporocysts were more elongated. Moreover, one side of the sporocysts was a bit flattened. In this feature sporocysts resembled *Goussia laureleus* (Molnár et Fernando, 1974), a parasite of the yellow perch, or *G. acerinae* (Pellérdy et Molnár, 1971), a parasite of the ruffe.

Discussion

Data from this study indicate that tadpoles of *Rana ridibunda* and *R. esculenta* are commonly infected with a coccidian parasite, which on the basis of similar hosts, locality, and oocyst morphology, appears identical to that which Nöller (1920) named *Eimeria neglecta*. The oocysts of this species are of characteristic *Goussia* type; therefore, the species has been transferred to the genus *Goussia*. Only a single other species of *Goussia* has been described from a non-fish host, a crocodile (Gardiner et al., 1986).

When Nöller (1920) described *G. neglecta* from tadpoles, he noted that the parasite disappeared from the host's intestine following metamorphosis, and he commented on the similarities between *G. neglecta* and coccidian parasites of fish. He also described the extremely thin oocyst walls and the presence of sporulated oocysts in yellow bodies.

Goussia neglecta greatly differs from the coccidia of adult frogs and represents a different type of infection. While frogs are infected by species of the genera *Eimeria* and *Isospora*, tadpoles carry *Goussia* infection, which is more similar to that of freshwater fishes than of adult frogs.

Meronts locating in the apical cytoplasma and comprising 8 to 16 merozoites as well as macrogamonts and young oocysts in the epithelial cells resembled *Goussia* infection of the common carp (Steinhagen, 1991) and silver carp (Molnár, 1976; Baska and Molnár, 1989) and unquestionably belong to *Goussia neglecta*. However, stages found in the nucleus of the epithelial cells seem to represent another coccidiosis. It is supposed that they might belong to a species infecting the frogs. Laveran and Mesnil (1902) have reported that *Eimeria ranarum* develops in the nucleus of the epithelium. Intranuclear development seems to be common in poikilothermic vertebrates. For instance, Finkelman and Paperna (1994*a, b*), studying the coccidia of skinks and lizards, have recently described some new species developing in that way.

One can only speculate how intranuclear coccidian stages can survive inside the metamorphosing animal. Another question that may arise is how the oocysts of *Goussia neglecta* survive after leaving tadpoles. It is well known that the oocysts of *Goussia* spp., having thin oocyst and sporocyst walls of low resistance, can survive only for a few weeks outside fish (Musselius et al., 1963). Therefore, it is probable that oocysts of *G. neglecta* can also survive only for a short time outside the host, and that they will not be able to infect a new tadpole generation a year later. A chance of the species to overwinter might be the survival of sporozoites in tubificid worms as has been proved by Steinhagen and Körting (1990) for *G. carpelli*. Nor can it be excluded, however, that the coccidia of tadpoles have a dormant stage in frogs, as do the merozoites of *Goussia subepithelialis* (Moroff et Fiebiger, 1905) in common carp (Marincek, 1973), and produce the infective stage in frogs a year later.

At the same time, the occurrence of intranuclear stages leads us to speculate that infection of frogs with *Eimeria*-type apicomplexans might start already in tadpoles, and can initiate an infection in young frogs. Developmental stages found in the nuclei of epithelial cells do not seem to belong to *G. neglecta;* rather, they appear to be part of the parasite fauna of adult frogs.

Acknowledgement

The author is thankful to Dr. S. S. Desser for his comments.

References

Baska, F. and Molnár, K. (1989): Ultrastructural observations on different developmental stages of *Goussia sinensis* (Chen, 1956) a parasite of the silver carp (*Hypophthalmichthys molitrix* Valenciennes, 1844). Acta Vet. Hung. **37**, 81–87.

Chen, G. J. and Desser, S. S. (1989): The Coccidia (Apicomplexa: Eimeriidae) of frogs from Algonquin Park, with description of two new species. Can. J. Zool. **67**, 1686–1689.

Dobell, C. C. (1909): Research on the intestinal protozoa of frogs and toads. Q. J. Microsc. Sci. **53**, 201–277.

Finkelman, S. and Paperna, I. (1994*a*): The endogenous development of three new intranuclear species of *Isospora* (Apicomplexa: Eimeriidae) from agamid lizards. Syst. Parasitol. **27**, 213–226.

Finkelman, S. and Paperna, I. (1994*b*): The endogenous development of two new species of *Isospora* (Apicomplexa: Eimeriidae) from skinks. Syst. Parasitol. **27**, 227–235.

Gardiner, C. H., Imes, G. D., Jacobson, E. R. and Foggin, C. M. (1986): Sporulated coccidian oocysts resembling *Goussia* Labbé, 1896 in the viscera of the Nile crocodiles. J. Wildlife Dis. **22**, 575–577.

Grassi, G. B. (1882): Intorno ad alcuni protisti endoparassitici ed appartenenti alle classi dei flagellati, lobosi, sporozoi e ciliati. Atti. Soc. Ital. Sci. Nat. Mus. Civ. Stor. Nat. Milano. **24**, 135–224.

Labbé, A. (1894). Recherches zoologiques et biologiques sur les parasites endoglobulaires du sang des vertébrés. Arch. Zool. Exp. Gen. **2** (Ser. 3), 55–258.

Laveran, M. A. and Mesnil, F. (1902): Sur deux coccidies intestinales de la *Rana esculenta*. C. R. Seances Soc. Biol. Ses Fil. **54**, 857–860.

Marincek, M. (1973): Dévelopment d' *Eimeria subepithelialis* (Sporozoa, Coccidia) parasite de la carpe. Acta Protozool. **19**, 197–215.

Molnár, K. (1976): Histological study of coccidiosis caused in the silver carp and the bighead by *Eimeria sinensis* Chen, 1956. Acta Vet. Hung. **26**, 303–312.

Musselius, V. A., Ivanova, N. S. and Laptev, V. I. (1963): On the coccidiosis of the common carp. Report One (in Russian). Tr. VNIIPRH **12**, 183–196.

Nöller, W. (1920): Zur Kenntniss der Coccidien des Wasserfrosches (*Eimeria neglecta* nov. spec.) (Befructung und Sporogonie von *Lankesterella*). Arch. Protistenkd. **41**, 176–180.

Pachinger, A. (1886): Mitteilung über Sporozoen. Zool. Anz. **9**, 471–472.

Steinhagen, D. (1991): Ultrastructural observations on merogonic and gamogonic stages of *Goussia carpelli* (Apicomplexa, Coccidia) in experimentally infected common carp *Cyprinus carpio*. Eur. J. Protistol. **27**, 71–78.

Steinhagen, D. and Körting, W. (1990): The role of tubificid oligochaetes in the transmission of *Goussia carpelli*. J. Parasitol. **76**, 104–107.

Upton, S. J. and McAllister, C. T. (1988): The *Coccidia* (Apicomplexa: Eimeriidae) of Anura, with descriptions of four new species. Can. J. Zool. **66**, 1822–1830.

Acta Veterinaria Hungarica 43 (2–3), pp. 277–286 (1995)

EFFECT OF EXPOSURE TO MALACHITE GREEN SOLUTION ON COMMON CARP FRY WITH *DACTYLOGYRUS VASTATOR* (MONOGENEA) INFECTION

K. MOLNÁR

Veterinary Medical Research Institute, Hungarian Academy of Sciences,
H–1581 Budapest, P.O. Box 18, Hungary

(Received December 5, 1994)

In five experiments, common carp (*Cyprinus carpio*) fry with *Dactylogyrus vastator* (Monogenea) infection of different severity, as well as parasite-free fry, were placed into aquaria containing malachite green solution of 0.5–10 mg/l concentration. Most of the fish died within 6 hours. The correlation between the time of death and helminth infection was studied. The experiments demonstrated that in malachite green solution the carp with the heaviest helminth infection died first while the less severely infected specimens survived for the longest time.

Key words: *Dactylogyrus vastator,* Monogenea, common carp, malachite green, survival

The effects of parasitic infections and their role in the host's death are difficult to assess. Parasites very often cause only subclinical infection which does not necessarily lead to the host's death. At the same time, living beings whose natural resistance has been impaired by parasitoses are unquestionably more susceptible to environmental stressors than those free from parasitic infection. This is especially true for fish which, besides parasitic infections, are exposed to a wide range of physical and chemical stressors in the aquatic environment. The interactive effects exerted on fish by environmental factors and parasitoses have been summarized by Overstreet (1994). Prior to this study, Molnár (1994) studied the effect of physical stressors, more closely of the reduced oxygen content of water, on common carp with *Dactylogyrus vastator* infection of different severity.

In addition to adverse physical effects, fish are exposed to a variety of chemical stressors such as the changes in water pH and ammonia concentration as a result of photosynthesis depending on the aquatic vegetation, hydrogen sulphide generated by anaerobic decomposition processes taking place in the mud, or the chemicals used for medication purposes, all of which factors adversely affect the survival chances of fish infected by parasites.

The objective of experiments conducted in a *D. vastator* model was to determine the response of common carp fry affected by parasitic infection of

0236–6290/95/$ 4.00 © 1995 *Akadémiai Kiadó, Budapest*

different severity to bathing in a slight overdose of malachite green solution, used as a therapeutic compound owing to its antiprotozoal effect.

Materials and methods

For the experiments, fertilized eggs derived from a single female fish were hatched in the laboratory, and were reared under sterile conditions, in tap-water, free of parasitic and bacterial infections and fed on *Artemia salina*. The fish measured 14–24 mm at the time of the experiment.

Common carp fry infected by *D. vastator*, derived from a fish farm, were used for the infection experiments. The fry were rendered free from ectoparasitic protozoans by 2×24-hour exposure to 0.1 mg/l malachite green solution. After this exposure fish remained infected only by *D. vastator* which laid hundreds of eggs. These fish were then removed from the aquarium, and 3–4 days later, when oncomiracidia had hatched and were swimming in the water, parasite-free fish were placed into the aquarium for different periods of time. The degree of infestation was checked by sacrificing some fish and counting the oncomiracidia that had lost their cilia and colonized the skin and gills. In each case an attempt was made to include in the sample fish with different severity of infection (ranging from a few to approx. one hundred helminths). The fry infected by the above method were drawn into experiments 4 to 6 days after infection, when the helminths had reached maturity (i.e. when their haptoral organs had developed and the first eggs appeared). In each experiment, 5–6 uninfected specimens were used besides infected fry.

Malachite green oxalate manufactured by Bayer AG (Germany) and widely used against protozoan parasites of fish in Hungary was used in the experiment. The fish were placed into aerated glass aquaria containing 4 litres of malachite green solution of a concentration that varied between 0.5 and 10 mg/l by experiment. Fish were removed from the aquaria immediately after death, and the helminths found on their gills were counted under a dissection microscope. At the start of the experiment the small and fragile fish often sustained mechanical injuries. Such injured fish specimens were excluded from the trial; this is why in the first experiments the number of fish deviated from the planned value. The size of fish, the temperature of the solution, the time that elapsed until death, and the number of parasites found were recorded in each experiment (Tables 1–5). The number of fish included in the different experiments and the applied concentrations of malachite green solution are also shown in the tables. Group averages were calculated by assigning the fish to groups of five or six in the order of their death. Statistical differences between the groups were evaluated by Student's *t* test on the basis of the mean worm burden.

Results

By the infection model applied, i.e. by decreasing or increasing the time of exposure, fish with *D. vastator* infection of different severity could be produced for the experiments. Shorter exposure in an infected aquarium resulted in lower while a longer exposure in higher numbers of *Dactylogyrus* oncomiracidia colonizing the gills of fish. As a result of infection, 1–96 oncomiracidia could be found on the experimental fish. The infection severity scale thus created was complemented by the addition of infection-free fish specimens as control.

A general trend observed during the experiments was that the fish groups dying first always proved to have the most severe *Dactylogyrus* infection. At the same time, a high variation was found within the different groups.

The first experiment (Table 1) can be considered practically unsuccessful, as the fish died within a very short time due to the high concentration of malachite green solution applied (10 mg/l). This experiment included 5 infection-free and 15 infected fish, 4 of which died of a mechanical injury immediately after the start of the experiment. Subsequently, four out of the survivors proved to be parasite-free, while the other fish specimens harboured 4–96 parasites. In this experiment, only the fish assigned to the first group showed a major difference from the other two groups in mean worm burden; however, not even this difference proved to be statistically significant.

Eighteen out of the 20 fish included in experiment 2 (Table 2), in which a malachite green concentration of 1 mg/l was applied, were evaluated. Two of them proved to be free of infection, while the others harboured 4–72 helminths. The order of groups ranked by the time of death was as expected; i.e. more severely infected fish died first; however, due to the fairly similar mean worm burdens the differences were not statistically significant.

In experiments 3 and 5 (Tables 3 and 5) there were no fish with mechanical injuries: all the 30 fish specimens drawn into both of these experiments were suitable for evaluation. The group ranking was as expected: a distinct correlation was seen between the time of death and the severity of infection. Although the difference between the neighbouring groups was not always statistically significant, between the non-neighbouring groups a statistically significant difference was a consistent finding.

In experiment 4 (Table 4), in which the fish were exposed to a malachite green solution of 0.5 mg/l concentration, also the fish belonging to the most severely infected group died first and those with the least severe infection survived for the longest time; however, the difference found between the groups was not statistically significant.

Table 1

Order of death of common carp fry with *Dactylogyrus vastator* infection of different severity in a malachite green solution of toxic concentration (10 mg/l)

Experiment 1	Group 1	Group 2	Group 3
No. of fish grouped in the order of death	1–5	6–10	11–16
Size of fish (cm)	1.5–1.7	1.5–1.7	1.5–1.7
Temperature during the experiment (°C)	24	24	24
Time to death	0:12–0:14	0:15–0:17	0:18–0:20
Worm burden, mean	30.4 ± 37.5	6.6 ± 9.0	10.2 ± 1.64
Range	4–96	0–18	0–12
		P > 0.165	P > 0.445
		P > 0.306	

Table 2

Order of death of common carp fry with *Dactylogyrus vastator* infection of different severity in a malachite green solution of toxic concentration (1 mg/l)

Experiment 2	Group 1	Group 2	Group 3
No. of fish grouped in the order of death	1–6	7–12	13–18
Size of fish (cm)	1.4–1.7	1.5–1.6	1.6–1.7
Temperature during the experiment (°C)	24	24	24
Time to death	3:30–3:50	3:55–4:25	4:30–4:40
Worm burden, mean	26.8 ± 25.2	18.0 ± 11.1	10.6 ± 9.8
Range	14–72	4–32	0–20
		P > 0.596	P > 0.219
		P > 0.130	

Table 3

Order of death of common carp fry with *Dactylogyrus vastator* infection of different severity in a malachite green solution of toxic concentration (1 mg/l)

Experiment 3	Group 1	Group 2	Group 3	Group 4	Group 5
No. of fish grouped in the order of death	1–6	7–12	13–18	19–24	25–30
Size of fish (cm)	1.4–1.8	1.5–1.7	1.5–1.7	1.3–1.8	1.4–1.8
Temperature during the experiment (°C)	24	24	24	24	24
Time to death	2:05–2:15	2:20–2:30	2:45–3:30	3:35–3:50	4:0–4:45
Worm burden, mean	45.5 ± 22.1	35.5 ± 32.1	28.8 ± 21.9	7.0 ± 6.9	0.66 ± 1.0
Range	24–72	2–78	8–62	0–16	0–2

$P > 0.635$ $P > 0.639$ $P < 0.061$ $P < 0.070$

$P > 0.333$ $P = 0.027$

$P = 0.14$

$P = 0.048$

$P = 0.058$

$P < 0.04$

Table 4

Order of death of common carp fry with *Dactylogyrus vastator* infection of different severity in a malachite green solution of toxic concentration (0.5 mg/l)

Experiment 4	Group 1	Group 2	Group 3	Group 4	Group 5
No. of fish grouped in the order of death	1–6	7–12	13–18	19–24	25–30
Size of fish (cm)	1.7–2	1.7–2	1.8–2	1.7–2	1.7–2
Temperature during the experiment (°C)	24	24	24	24	24
Time to death	2:50–3:00	4:00–4:20	4:30–4:50	5:10–5:50	6:00–killed
Worm burden, mean	32.1 ± 21.0	26.8 ± 23.4	12.8 ± 16.3	16.5 ± 12.4	10.6 ± 7.1
Range	0–64	0–56	0–40	4–40	0–18

P = 0.627 P = 0.360 P = 0.703 P = 0.265

P = 0.550 P = 0.736

P = 0.394

P = 0.172

P = 0.174

P = 0.098

Table 5

Order of death of common carp fry with *Dactylogyrus vastator* infection of different severity in a malachite green solution of toxic concentration (1 mg/l)

Experiment 5	Group 1	Group 2	Group 3	Group 4	Group 5
No. of fish grouped in the order of death	1–6	7–12	13–18	19–24	25–30
Size of fish (cm)	2.2–2.4	2.2–2.4	2.2–2.4	2.2–2.3	2.0–2.4
Temperature during the experiment (°C)	24	24	24	24	24
Time to death	1:25–3:0	3:5–4:10	4:15–5:0	5:05–5:20	5:30–killed
Worm burden, mean	61.6 ± 32.4	34.0 ± 22.2	10.3 ± 11.4	9.66 ± 8.11	0.3 ± 1.16
Range	1–96	0–55	0–26	0–20	0–3

P = 0.081 P = 0.067 P = 0.919 P = 0.045

P = 0.019 P = 0.081

P = 0.071

P = 0.016

P = 0.015

P = 0.006

Discussion

The impairing effect exerted by parasites on the host's resistance is a long known fact amply documented in numerous textbooks. It is an incontrovertible fact that this resistance-impairing effect of parasitic organisms, making the host less resistant to biotic and abiotic environmental stressors, is much more important than their direct pathogenic effect. At the same time, the number of works addressing this subject in an experimental setting is still very low.

A relationship between parasitism and the stress due to water pollution caused by chemicals has been suggested by several authors. Some publications discuss the correlation between pollution and parasitism in general (Sinderman, 1979; Möller, 1986; Overstreet, 1988), while others highlight an interaction between a specific chemical pollutant and a parasite. The chlorinated effluents of bleached kraft pulp mills are especially favourable for parasite burden. According to Lehtinen et al. (1984), the gills of fish kept in the polluted effluents of such mills were colonized by a higher number of *Trichodina* organisms than those of fish kept in clear water. A similar observation was made by Thulin et al. (1988), who recorded a higher incidence of monogeneans on the gills of *Rutilus rutilus* living in chlorinated effluents. Similarly, the losses caused by monogeneans were facilitated by the oil pollution of sea-water (Khan and Kiceniuk, 1988). Under experimental conditions, the above authors observed permanent colonization of the gills of cod by *Gyrodactylus* spp. in an aquarium containing water polluted by petroleum aromatic hydrocarbons. Kuperman (1992) put the correlation between anthropogenic water pollution and parasitoses in a unique new light, stressing that pollution leads to an increase in the number of parasites, and demonstrating structural anomalies in *Diplozoon paradoxum* specimens as a result of pollution. Of the heavy metals, the role of copper sulphate, a compound often used for medication purposes, has been emphasized by Ewing et al. (1982): in *Ichthyophthirius multifiliis* infection copper sulphate causes epithelial hyperplasia of the gills. Boyce and Yamada (1977) reported that sockeye salmon infected by *Eubothrium salvelini* are much less resistant to zinc as a water pollutant than noninfected specimens of the same species. The elevated cadmium level of water may also aggravate the pathological effects exerted by parasites. Mohan and Sommerville (1989) demonstrated that the presence of that heavy metal in the water markedly enhanced the intensity of *Ichthyophthirius multifiliis* infection. According to Sövényi and Szakolczai (1993), cadmium has an immunosuppressive effect whereby it helps biotic factors manifest their pathogenicity. Malachite green, which is often used as a therapeutic agent, is also known to provoke major changes in the fish organism even in therapeutic doses. For instance, Margaritov (1982) reported that malachite green, when used as a 5-hour antiparasitic bath in a concentration of 0.5 mg/l identical with that used in this study, markedly affected

the size and age composition of the peripheral blood erythrocytes of the common carp.

The results of this study support the above-cited data of the literature and indicate that a positive correlation exists between the rapidity of death and parasitic infection also in the case of lethal chemical stress, in the same way as has been demonstrated in earlier experiments for a physical stress such as decreased water oxygen content (Molnár, 1994). According to Uspenskaya (1961), 40–60 *D. vastator* specimens can kill common carp fingerlings also without any other stressor. As found by the present author, marked gill damage develops only after 1–2 weeks of parasitic infection; therefore, in these experiments carried out 4–6 days after infection a direct lethal effect of the parasites could not yet manifest itself. It is obvious that exposure to sublethal concentrations of malachite green was responsible for the mortality; however, parasitic infection markedly influenced the time that elapsed to death, and it was a consistent finding that fish infected by higher numbers of parasites died first.

Acknowledgements

The author wishes to thank Ms Emese Papp for technical help in the implementation of the experiments. Thanks are due to A. Gubányi (Hungarian Natural History Museum, Zoological Department) for statistical evaluation of the data.

This work was supported by the National Research Fund (OTKA, project no. 1985) of Hungary.

References

Boyce, N. P. and Yamada, S. B. (1977): Effects of a parasite, *Eubothrium salvelini* (Cestoda: Pseudophyllidae), on the resistance of juvenile sockeye salmon, *Oncorhynchus nerca*, to zinc. J. Fish. Res. Board Canada **34,** 706–709.

Ewing, M. S., Ewing, S. A. and Zimmer, M. A. (1982): Sublethal copper stress and susceptibility of channel catfish to experimental infections with *Ichthyophthirius multifiliis*. Bull. Environ. Contam. Toxicol. **28,** 674–681.

Khan, R. A. and Kiceniuk, J. W. (1988): Effect of petroleum aromatic hydrocarbons on monogeneids parasitizing Atlantic cod, *Gadus morhua* L. Bull. Environ. Contam. Toxicol. **41,** 94–100.

Kuperman, B. I. (1992): Parasites of fish as bioindicators for pollution of waters (in Russian). Parazitologiya **26,** 479–482.

Lehtinen, K.-J., Notini, M. and Landner, L. (1984): Tissue damage and parasite frequency in flounders, *Platichthys flesus* (L.) chronically exposed to bleached kraft pulp mill effluents. Ann. Zool. Fenn. **21,** 23–28.

Margaritov, N. (1982): Influence of some antiparasitic baths on the size and age composition of the peripheral blood erythrocytes of the common carp (in Bulgarian, with English abstract). Annuaire L'Univ. Sofia **75,** 82–91.

Mohan, C. V. and Sommerville, C. (1989): Interaction between parasitic infection and metal toxicity in fish; an experimental study. Proc. XIVth Symp. Scand. Soc. Parasit., Information **20,** 62.

Molnár, K. (1994): Effect of decreased water oxygen content on common carp fry with *Dactylogyrus vastator* (Monogenea) infection of varying severity. Dis. aquat. Org. **20,** 153–157.

Möller, H. (1986): Pollution and parasitism in the aquatic environment. Int. J. Parasitol. **17,** 353–361.

Overstreet, R. M. (1988): Aquatic pollution problems, Southeastern U.S. coast: histopathological indicators. Aquat. Toxicol. **11,** 213–239.

Overstreet, R. M. (1994): Parasitic diseases of fishes and their relationship with toxicants and other environmental factors. In: Couch, J. A. and Fournie, J. W. (eds) Pathobiology of Marine and Estuarine Organism. CRC Press, Boca Raton, pp. 111–156.

Sinderman, C. J. (1979): Pollution-associated diseases and abnormalities of fish and shellfish: a review. Fish. Bull. **76,** 717–749.

Sövényi, J. and Szakolczai, J. (1993): Studies on the toxic and immunosuppressive effect of cadmium on the common carp. Acta Vet. Hung. **41,** 415–426.

Thulin, J., Höglund, J. and Lindesjöö, E. (1988): Diseases and parasites of fish in a bleached kraft mill effluent. Water Sci. Technol. **20,** 179–180.

Uspenskaya, A. A. (1961): Effect of *Dactylogyrus vastator* Nybelin, 1924 on the organism of the common carp (in Russian). Zool. Zh. **10,** 7–12.

Acta Veterinaria Hungarica 43 (2–3), pp. 287–289 (1995)

PREVALENCE OF BOTFLY LARVAE AND LICE IN STUDS OF NORTH CAUCASUS (STAWROPOL COUNTY, RUSSIA)

B. EGRI[1], P. SÁRKÖZY[2] and GY. BÁNHIDY[2]

[1]Department of Animal Physiology and Animal Health, Pannon University for Agricultural Sciences, H–9200 Mosonmagyaróvár, Vár u. 4, Hungary;
[2]Sanofi-Chinoin Pharmaceutical and Chemical Works, H–1045 Budapest, Tó u. 1–5, Hungary

(Received January 19, 1995)

From 14 to 17 April 1990 and from 12 to 21 May 1993 a total of 136 Caucasian (Donish, Karabakh, Kabardin, local halfbred) and 26 thoroughbred stud-horses were examined for ectoparasites in studs of Stawropol County, Russia. A total of 4054 botfly larvae were identified from 9700 faecal samples collected during a period of 3–4 days after oral treatment with Eqvalan (MSD), Strongid Plus (Pfizer) and Vermitan Plus (Sanofi-Chinoin). The species distribution was: *Gasterophilus intestinalis* 84.53%, *G. nasalis* 10.41%, *G. haemorrhoidalis* 3.62%, *G. inermis* 1.41%. *Gasterophilus nasalis* and *G. inermis* had not been recorded in the North Caucasian region previously. One hundred and fourteen (of the 136) halfbred and 26 thoroughbred horses were examined for ectoparasites, and all were found to be infested with biting lice (*Werneckiella equi equi*) and sucking lice (*Haematopinus asini*).

Key words: Horse, prevalence, ectoparasite, botfly larva, louse, North Caucasus

As data on the prevalence of botfly larvae and lice of horses in the North Caucasian region were scarce and incomplete (Grunin, 1953; Nepoklonow et al., 1980) the aim of the present trials was to collect further information on the occurrence of these ectoparasites of working horses in studs.

Materials and methods

Between 14–17 April 1990 and 12–17 May 1993, a total of 136 Caucasian (Donish, Karabakh, Kabardin and local halfbred) horses from different Caucasian localities, and 26 thoroughbred stud-horses of different age and sex were examined for the occurrence of ectoparasites in the studs of Stawropol County, Russia. In the previous years, all of the horses had been kept on the same two pastures in the grazing season. Botfly eggs laid on the haircoat of horses were observed in late

0236–6290/95/$ 4.00 © 1995 *Akadémiai Kiadó, Budapest*

summer. Nits, nymphal stages and adults of lice were found on the head, neck, breast (!) and scapula (!) in May 1993.

During the experiments, the horses were kept on deep litter under loose housing conditions. They were fed corn silage, 1.1 kg corn seed/horse/day and, from the 3rd day of the second trial, rape *ad libitum.* The stallions received also straw *ad libitum.*

The 136 experimental animals in different studs were treated with the preparations shown in Table 1.

The total number of botfly larvae spontaneously expelled in the faeces of individual horses was determined. The examination of faeces was carried out at 2-h intervals for 3 days (Egri, 1989), or from 8:00 a.m. till 8:00 p.m. every 2 h for 4 days, and the number of *Gasterophilus* larvae was recorded.

Larvae were identified using a Wild M420 type photomicroscope. For the identification of botfly larvae the keys of Grunin (1953) and Zumpt (1965), while for that of lice the keys of Zlotorzycka et al. (1974) were used.

Table 1

Diagnostic preparations used in the trials against larvae of *Gasterophilus* spp.

Preparations (paste)	Dose of active ingredients	Number of treated animals
Eqvalan (MSD) 1 tube/600 kg b.w.	0.2 mg/kg b.w.	49
Vermitan® Plus (Sanofi-Chinoin) 1 tube/500 kg b.w. (Egri and Sárközy, 1994)	10 mg albendazole/kg b.w. 1.5 mg cypermethrin (transisomers) /kg b.w.	79
Strongid Plus (Pfizer) 1 tube/550 kg b.w.	6.6 mg/ml pyrantel pamoate/kg b.w. 30 mg/ml trichlorfon/kg b.w.	8

Results and discussion

Before this study, only a single report had been known on the prevalence of different species of horse bots in the desert zones at the Caspian Sea (Rastegaev, 1983). This region lies approximately 300–350 km south-southeast of the territory where the present investigations were carried out. Our experimental data show that the stud-horses were extensively infested with botfly larvae, and the use of larvicide preparations enabled us to collect data on their species composition.

In the present study, 9700 spontaneously excreted faecal samples were examined. From these excrements a total of 4054 *Gasterophilus* larvae were col-

lected. The per cent distribution of the species identified was as follows: *G. intestinalis* 84.53%, *G. nasalis* 10.41%, *G. haemorrhoidalis* 3.62%, and *G. inermis* 1.41%. The intensity of infestation ranged from 26 to 35 larvae per animal. One mare had to be emergency slaughtered on the 2nd day of treatment because of colic. At postmortem examination, 175 *Gasterophilus* larvae were found in the stomach and in the first part of the duodenum.

Gasterophilus nasalis and *G. inermis* had not been recorded previously in the North Caucasian region.

In the stud of the Kaz'minskoie hamlet 114 out of 136 halfbred (local races) and 26 thoroughbred horses were examined for ectoparasites. All (100%) of these animals were infested with biting lice (*Werneckiella equi equi*) and sucking lice (*Haematopinus asini*).

References

Egri, B. (1989): Method for evaluating the larvicidal efficacy of Strongid Plus (Pfizer) and Eqvalan (MSD) paste against gasterophilosis. Parasit. Hung. **22,** 109–114.

Egri, B. and Sárközy, P. (1994): On occurrence of some ectoparasites in studs of North Caucasus. 8th ICOPA, Izmir. Abstracts, Vol. 2, 317.

Grunin, K. J. (1953): Larvae of Bots of Domesticated Animals (in Russian). Izd. Akad. Nauk SSSR, Moscow-Leningrad. 95 pp.

Nepoklonow, A. A., Hiepe, Th., Splissteser, H. and Dorz, C. (1980): Diseases of Animals Caused by Bots (in Russian). Kolos, Moscow. 255 pp.

Rastegaev, Yu. M. (1983): Ecology and control of *Rhinoestrus* and *Gasterophilus* in horses in desert zones around the Caspian Sea (in Russian). In: Nepoklonow, A. A. (ed.) Veterinary Entomology and Acarology (in Russian). Kolos, Moscow, 335 pp.

Zlotorzycka, J., Eichler, W. and Ludwig, H. W. (1974): Taxonomie und Biologie der Mallophagen und Läuse mitteleuropäischer Haus- und Nutztiere. VEB G. Fischer Verlag, Jena. pp. 15–16, pp. 134–136.

Zumpt, F. (1965): Myiasis in Man and Animals in the Old World. Butterworths, London, 267 pp.

Acta Veterinaria Hungarica 43 (2–3), pp. 291–296 (1995)

BETAMETHASONE AND ADULT RAT LUNG SURFACTANT LIPIDS

S. STETTNER[1] and A. LEDWOŻYW[2]

[1]Ist Clinic of General Surgery, Medical Academy, Jaczewskiego 8, 20–950 Lublin, Poland; [2]Department of Pathophysiology, Agricultural Academy, Lublin, Poland

(Received February 22, 1995)

Betamethasone (2 mg/kg/day) was administered to adult male rats (n = 15) for one week by daily subcutaneous injections. Thereafter the lungs were lavaged and lamellar bodies (intracellular surfactant storage granules) were isolated from the tissue. Lipid analyses showed two- to fourfold higher amounts of total phospholipids and disaturated phosphatidylcholine compared with the control. These changes were not found in the kidney liver and were not present in plasma membrane, mitochondrial or microsomal fractions from lungs. It was concluded that glucocorticoids play a significant effect in increasing the lung surfactant lipid pools of adult rat lungs by changing the phospholipid content of lamellar bodies.

Key words: Glucocorticoids, lamellar bodies, type II cells, phospholipids

Abundant evidence has been presented to demonstrate that accelerated lung epithelial cell maturation and precocious production of lamellar bodies accompany the prenatal administration of glucocorticoids to mammalian fetuses (Kikkawa et al., 1971; Kotas and Avery, 1971).

Clinically, benficial effects of glucocorticoids on adult lung surfactant have not been found as yet. Abe and Tierney (1977) administered 2 mg/kg/day hydrocortisone to adult male rats and found a 25% increase in homogenized whole lung saturated phsophatidylcholine after 7 days of treatment.

Liebowitz et al. (1984) reported that adrenalectomy resulted in a decrease of the extracellular phospholipid content in adult rat lung, which could be prevented by concurrent hydrocortisone injections.

Ballard (1977) reviewed the data indicating glucocorticoid binding to lung tissue and demonstrated that dexamethasone was bound with a higher affinity than was hydrocortisone. Beer et al. (1983) showed that [^{3}H]-dexamethasone binding could be demonstrated in type II cell nuclei and other lung cells in adult mouse lungs. *In vitro* culture of lung type II cells has generally provided evidence of a trophic influence of glucocorticoids on lamellar body content.

0236–6290/95/$ 4.00 © 1995 *Akadémiai Kiadó, Budapest*

Overall, the reports available suggest only amodest effect of glucocorticoids on adult lung type II cells. The present study was undertaken to determine the effects of betamethasone on adult rat lung subcellular lipid profiles.

Materials and methods

Adult Wistar male rats of an average body weight of 310 g were used throughout this study. They were held for 48 h and then given betamethasone (9-fluor-11β,17,21-trihydroxy-16β-methylpregna-1,4-dien-3,20-dione, Betapred, Glaxo, UK, 4 mg/ml, subcutaneously). Control animals were given equal volumes of diluent (1 mg sodium sulfite, 19.4 mg sodium citrate, 0.01 ml benzoyl alcohol/ml, pH = 7.0–8.5) by daily subcutaneous injections. Food and water were provided *ad libitum* and light-dark cycles were 12 h.

At the end of the injection schedules the animals were given a lethal dose of pentobarbital sodium (i.p). The abdomen was opened widely and the abdominal aorta cut. After removal the lungs were weighed and then lavaged 10 times with 10 ml each of 0.45% sodium chloride solution (4 °C). In some experiments (n = 18) the kidneys and liver were also removed.

Lipid extractions from lung tissues, lavage fluid, kidney tissue and liver were done according to Bligh and Dyer (1959). Saturated phosphatidylcholine (DSPC) was isolated according to Maon et al. (1976). Phospholipids were isolated on thin-layer chromatography plates (silica gel G-60 precoated plates, E. Merck, Darmstadt, Germany) according to Touchstone et al. (1980). Phosphorus was quantitated according to Bartlett (1959) and phospholipid amounts were calculated assuming 4% phosphorus content.

Protein was measured by the method of Lowry et al. (1951) using bovine serum albumin as standard. DNA was determined as described by Schneider (1957).

A lamellar body enriched fraction was obtained from homogenized lung tissue by the method of Duck-Chong (1978). Subcellular fractions of lung tissue homogenates were obtained by differential centrifugation according to Van den Bosch and de Jong (1975). After disruption in a Potter-Elvejhem homogenizer, tissue homogenates were centrifuged at 2000 *g* for 5 min to obtain a plasma membrane plus nuclear fraction. Mitochondria were isolated by cetrifugation at 20,000 *g* for 10 min. The final supernatant was centrifuged at 110,000 *g* for 60 min to isolate a microsomal fraction.

Contamination of the lamellar body enriched fraction by plasma membrane, mitochondria and microsomes was assessed by marker enzyme analysis. Plasma membranes were identified by 5'-nucleotidase (EC 3.6.3.5) according to Gentry and Olsson (1975), mitochondria by succinate: cytochrome c reductase (EC 1.3.99.1) and microsomes by rotenone-insensitive NADH: cytochrome c

reductase (EC 1.6.2.3) (Sotocassa et al., 1967). Total activity for a given marker enzyme was assumed to be the sum of the activity (μmole/min/mg protein × mg total protein) in the isolated organelle fraction. The amount of each enzyme in the lamellar body fraction was expressed as a percentage of total activity.

Significant differences between groups were tested by the procedure of Duncan for multiple group comparisons (1955), by Dunnett's procedure (1955) for multiple comparisons to a control, or Student' *t*-test. Significance was set at $p < 0.05$.

Results

The lung effects of betamethasone administered at 2 mg/kg/day for 7 days are given in Table 1. Food consumption of the control rats was 28 ± 1 g/animal/day and that of the betamethasone-treated rats was 22 ± 1 g/animal/day. There was a substantoial catabolic effect of the glucocorticoid as evidenced by the weight loss in the treated animals, compared with weight gain of the control animals. The 15% lower wet lung weight in the treated group was accompanied by a nonsignificantly lower lung DNA. Lung total protein did not change. There was a doubling of lung tissue total phospholipid (PL). Whole lung DSPC was 36% of total phospholipids in the control and 42% in the treated animals.

Table 1

Effect of betamethasone on lung tissue composition

	Control	Betamethasone
Body weight, g (day 0)	274 ± 4 (12)	275 ± 3 (19)
(day 7)	328 ± 5 (12)	244 ± 5 (19)*
Lung wet weight, g	1.50 ± 0.09 (12)	1.21 ± 0.05 (19)*
Lung DNA, mg/g lung	6.2 ± 0.7 (12)	6.0 ± 0.4 (19)
Lung protein, mg/g lung	77 ± 4 (12)	80 ± 5 (16)
Lung phospholipid, mg/g lung	11.6 ± 0.2 (13)	21.5 ± 0.5 (15)*
Lung DSPC, mg/g lung	3.9 ± 0.6 (13)	9.1 ± 0.6 (15)*

Values are mean ± S.D. for the number of animals given in parentheses. DSPC: disaturated phosphatidylcholine. *P < 0.05 vs. control

Table 2 shows the lamellar body fraction and lavage fluid results after betamethasone treatment. There was a much larger percentage increase in DSPC from the lamellar body fraction than from lung tissue and a substantial increase in the alveolar phospholipid pool size was also observed. In addition, the DSPC-to-protein ratio in lamellar bodies and in lavage increased significantly.

Table 2

Effect of betamethasone on lamellar body and lavage surfactant lipids

	Control	Betamethasone
Lamellar body fraction		
PL, mg/g lung	0.90 ± 0.11	4.62 ± 0.67*
DSPC, mg/g lung	0.52 ± 0.07	2.80 ± 0.24*
Protein, mg/g lung	0.20 ± 0.01	0.41 ± 0.06*
Lavage		
PL, mg/g lung	4.3 ± 0.4	8.1 ± 0.5*
DSPC, mg/g lung	1.1 ± 0.1	3.0 ± 0.3*
Protein, mg/g lung	5.4 ± 0.5	12.0 ± 1.2*

Values are mean ± S.D. (n = 16). PL: total phospholipids; DSPC: disaturated phosphatidylcholine. *P < 0.05 vs. control

The percentage distribution of phospholipid classes from lamellar bodies is given in Table 3. There were no significant differences with the exception of phosphatidylglycerol.

Table 3

Effect of betamethasone on lamellar body phospholipid classes

	Control	Betamethasone
Lysophosphatidylcholine	1.5 ± 0.4	1.7 ± 0.5
Sphingomyelin	1.3 ± 0.3	1.6 ± 0.4
Phosphatidylcholine	80.3 ± 2.3	82.0 ± 2.0
Phosphatidylinositol + phosphatidylserine	3.5 ± 0.4	4.0 ± 0.5
Phosphatidylethanolamine	2.1 ± 0.5	2.3 ± 0.6
Phosphatidylglycerol	6.4 ± 0.7	5.2 ± 0.9*
Remainder	ad 100	ad 100

Values are mean ± S.D. (n = 16) given in %. *P < 0.05 vs. control

Table 4 shows that the betamethasone effect on whole lung tissue was not seen in lipid extracts of liver or kidney tissue and is organ specific.

Phospholipid amounts in lung subcellular fractions other than lamellar bodies did not change (Table 5). Thus, the increase in phospholipid content was limited to lung tissue and extracellular pools specifically associated with surfactant (lamellar bodies and lavage):

Table 4

Effect of betamethasone on homogenized whole tissue disaturated phosphatidylcholine (μg DSPC/g tissue)

	Control	Betamethasone
Liver	1640 ± 210	1800 ± 190
Kidney	1850 ± 180	2100 ± 210
Lung	4200 ± 420	9760 ± 230*

Values are mean ± S.D. *P < 0.01 vs. control (n = 18)

Table 5

Effect of betamethasone on phospholipid content (μg PL/lung) of lung subfractions

	Control	Betamethasone
Plasma membrane	810 ± 90	830 ± 80
Mitochondria	980 ± 80	910 ± 70
Microsomes	1100 ± 140	1190 ± 170
Lamellar bodies	1300 ± 170	5300 ± 210*

Values are mean ± S.D. (n = 15). *P < 0.01 vs. control (n = 18)

Discussion

Betamethasone (2 mg/kg/day for 7 days) affected lung lipids of rats with a doubling of lung tissue phospholipid pools. A lamellar body enriched fraction showed even greater increases, almost fivefold, in total phospholipid and in saturated DSPC content. Constitutive changes in lamellar body composition were shown by the doubling of the DSPC-to-protein ratio.

We interpret our results to suggest specifically an increase in lamellar bodies, representing an intracellular storage pool. The extracellular compartment lipid content was also changed by betamethasone as shown by the lavage data in Table 2.

The alterations in lung lipids were limited to subcellular and lavage fractions associated with surfactant production. No other subcellular fractions demonstrated any change in DSPC. Liver and kidney whole tissue phospholipids did not change after betamethasone treatment. Thus, the effects on DSPC were organ specific and apparently limited to the surfactant pool of the lung.

We have demonstrated that schedules of betamethasone used clinically in some human disease states can significantly affect adult rat lung lamellar body

composition. Since betamethasone has a long biological half-life, we estimate that the 2 mg/kg dose might produce tissue concentrations as high as 5×10^{-6} M, even without active uptake or concentratione in lung tissue. Thsi level suggests that the steroid effect might not be entirely receptor mediated, although the lung tissue has numerous high-affinity betamethasone receptors (Ballard, 1977). The use of such high doses in humans is usually limited to the treatment of disorders such as life-threatening cerebral oedema or acute respiratory insufficiency. These considerations may also explain the smaller effects reported by Abe and Tiernea (1977). They used 8 mg/kg/day of hydrocortisone; our doses would be an approximate equivalent of 54 mg/kg/day of hydrocortisone.

We conclude that glucocorticoids alter lamellar body and lavage lipid content in adult rat lungs. The lamellar body effect is likely to be due to an increased phospholipid content per lamellar body.

References

Abe, M. and Tierney, D. F. (1977): Lung lipid metabolism after 7 days of hydrocortisone administration to adult rats. J. Appl. Physiol. **42,** 202–205.

Ballard, P. L. (1977): Glucocorticoid receptors in the lung. Fed. Proc. **36,** 2660–2665.

Bartlett, G. R. (1959): Phosphorus assay in column chromatography. J. Biol. Chem. **234,** 466–468.

Beer, D. G., Cunha, G. R. and Malkinson, A. M. (1983): Autoradiographic demonstration of the specific binding and nuclear localization of [^{3}H]-dexamethasone in adult mouse lung. Lab. Invest. **49,** 725–734.

Bligh, E. G. and Dyer, W. J. (1959): A rapid method of total lipid extraction and purification. Can. J. Biochem. **37,** 511–517.

Duck-Chong, C. J. (1978): The isolation of lamellar bodies and their membranous content from rat lung, tracheal fluid and human amniotic fluid. Life Sci. **22,** 2025–2030.

Duncan, D. B. (1955): Multiple range and multiple F tests. Biometrics **11,** 1–42.

Dunnett, C. W. (1955): A multiple comparison procedure for comparing several treatments with a control. J. Am. Stat. Assoc. **50,** 1096–1121.

Gentry M. K. and Olsson, R. A. (1975): A simple, specific, radioisotopic assay for 5'-nucleotidase. Anal. Biochem. **64,** 624–627.

Kikkawa, Y., Kaibara, M., Motoyama, E. K., Orzalesi, M. M. and Cook, C. D. (1971): Morphologic development of fetal rabbit lung and its acceleration with cortisol. Am. J. Pathol. **64,** 423–442.

Kotas, R. V. and Avery, M. E. (1971): Acceleration of appearance of pulmonary surfactant in the fetal rabbit. J. Appl. Physiol. **30,** 358–361.

Liebowitz, D., Massaro, G. D. and Massaro, D. (1984): Adrenalectomy and surfactant in adult rats. J. Appl. Physiol. **56,** 564–567.

Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951): Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193,** 265–275.

Mason, R. J., Nellenbogen, J. and Clements, J. A. (1976): Isolation of disaturated phosphatidylcholine with osmium tetroxide. J. Lipid Res. **17,** 281–284.

Schneider, W. C. (1957): Determination of nucleic acids in tissue by pentose analysis. Methods Enzymol. **3,** 680–684.

Sotocassa, G. I., Kuylenstiena, B., Ernster, L. and Berstand, A. (1967): An electron transport system associated with the outer membrane of liver mitochondria. J. Cell. Biol. **32,** 415–438.

Touchstone, J. C., Chen, J. C. and Beaver, K. M. (1980): Improved separation of phospholipids in thin layer chromatography. Lipids **15,** 61–62.

Van den Bosch, H. and de Jong, J. G. N. (1975): Studies on lysophospholipases IV. The subcellular distribution of two lysolecithin hydrolyzing enzymes in beef liver. Biochim. Biophys. Acta **398,** 244–257.

Acta Veterinaria Hungarica 43 (2–3), pp. 297–302 (1995)

THE EFFECT OF BETAMETHASONE ON PHOSPHATIDYLCHOLINE SPECIES COMPOSITION IN FETAL RAT LUNGS

S. STETTNER[1] and A. LEDWOŻYW[2]

[1]Ist Clinic of General Surgery, Medical Academy, Jaczewskiego 8, 20–950 Lublin, Poland; [2]Department of Pathophysiology, Agricultural Academy, Lublin, Poland

(Received February 22, 1995)

After betamethasone treatment, a significant diminution of the monoenoic phosphatidylcholine 32 carbon species (palmitoyl-palmitoleyl phosphatidylcholine and palmitoleyl-palmitoylphosphatidylcholine) and the phosphatidylcholine 34 carbon species (primarily palmitoyl-oleoyl-phosphatidylcholine) could be demonstrated both in absolute and relative terms, while the palmitic acid portion in the phosphatidylcholine fatty acids was nearly unchanged. This is consistent with a significant reduction of the palmitoleic and oleic acid portions of the total phosphatidylcholine fatty acids in the lungs of rat fetuses.

Key words: Betamethasone, lung, rat, fetal, phosphatidylcholine species

Acceleration of fetal pulmonary maturation processes in cases of imminent premature delivery or when progressive intrauterine damage makes premature delivery necessary represents a yet unsolved problem.

Liggins (1969) as well as Liggins and Howie (1972) were the first to report on the acceleratory effect of maternally administered glucocorticoids on fetal lung maturation. These findings were subsequently substantiated by numerous studies on various species. A similar effect on fetal lung maturation has also been demonstrated for several other hormones: adrenocorticotropic hormone (Sundell et al., 1979), 17β-estradiol (Possmayer et al., 1981), and prolactin (Hamosh and Hamosh, 1977). Animal experiments and clinical observations suggest that β-adrenergic agonists such as isoxsuprine (Kanjanapone et al., 1980) and hexoprenaline (Lipshitz et al., 1981), and cholinergic agonists such as oxotremorine (Abdellatif and Hollingsworth, 1980) also promote fetal lung maturation.

The effect of betamethasone on phosphatidylcholine species composition in fetal rat lungs was studied in this work.

Materials and methods

Thirty gravid Wistar rats with an average body weight of 300 g and an expected gestation period of 23 days were randomized, divided into two groups and

0236–6290/95/$ 4.00 © 1995 *Akadémiai Kiadó, Budapest*

treated from the 17th to the 20th day of gestation with 1 ml/day physiological saline (controls to equalize trauma), or 0.3 mg/kg betamethasone (9-fluor-11β, 17,21-trihydroxy-16β-methylpregna-1,4-dien-3,20-dione, Betapred, Glaxo, UK, 4 mg/ml) administered intraperitoneally. Delivery by Caesarean section took place on the 21st day. Laparotomy of the dams was performed by making a longitudinal abdominal incision. During hysterectomy, both uterine horns were opened along their full length. The fetuses, not allowed to breathe, were thoracotomized by means of a parasternal incision. The fetal lungs were then grouped according to litter in order to exclude station-related differences.

The fetal lungs were homogenized in 40 volumes of chloroform/methanol (2:1 v/v) and extracted overnight under nitrogen (oxygen free). The tissue lipid extracts were washed and the phospholipids were assayed by Bartlett's method (1959).

1,2-Dipalmitoyl-sn-glycero-3-phosphocholine was assayed as a diacylglycerol trimethylsilyl ether derivative by gas-liquid chromatography, using 1,2-dimyristoyl-sn-glycer-3-phosphocholine serving as standard (Lohninger and Nikiforov, 1980).

Aliquots of the samples were applied as bands on thin layer chromatographic plates (silica gel G-60, E. Merck, Darmstadt, Germany). The plates were developed in a chloroform/methanol/1% potassium chloride mixture (43:47:4 by volume) and dried, and the 3-sn-phosphatidylcholine fraction was removed from the plates. The 1,2-diacyl-sn-glycerols of 3-sn-phosphatidylcholine were obtained by digestion with phospholipase C from *Bacillus cereus* (Sigma, St. Louis, USA). The trimethylsilyl ethers of 1,2-diacyl-sn-glycerols were prepared by the reaction with pyridine/hexamethyldisilazane/chlorotrimethylsilane (12:5:2 by volume). The t-butyldimethylsilyl ethers of diacylglycerols were prepared by the method of Myher et al. (1978) by reaction with t-butyldimethylsilyl chloride. The resulting trimethylsilyl and t-butyldimethylsilyl ether solutions were used directly for subsequent gas-liquid chromatographic analyses. Phosphatidylcholine fatty acids were determined as methyl ester derivatives by gas-chromatographic analysis.

Statistical comparisons between the control and the treated group were made by Dunnett's test for multiple comparisons.

Results and discussion

The phophatidylcholine 32 carbon monoenoic species can be characterized as 16:0/16:1-phosphatidylcholine (16:0/16:1-PC) and 16:1/16:0-phosphatidylcholine (16:1/16:0-PC) (Lohninger and Nikiforov, 1980; Nikiforov et al., 1982). A relatively high portion of 16:1/16:0-PC is probably characteristic of the fetal

lung as compared to the adult lung, since analogous patterns have also been found in human amniotic fluid. Table 1 shows the percentage portion of the 16:1/16:0-PC in the phosphatidylcholine species of the fetal lung. 16:0/16:1-PC is significantly lower in the steroid-treated group than in the controls. These differences in phosphatidylcholine species composition are also manifested in quantitative terms. These findings were confirmed by analysis of the phosphatidylcholine fatty acid composition.

Table 1

Proportion of monounsaturated 32-carbon phosphatidylcholine species in fetal rat lung phosphatidylcholine following maternal betamethasone treatment

	16:0/16:1	16:1/16:0
Control	4.65 ± 0.33	11.92 ± 0.86
Betamethasone, mg/g dry weight	3.00 ± 0.42*	8.00 ± 1.05*
Control	1.25 ± 0.10	2.90 ± 0.22
Betamethasone, mg/g dry weight	0.88 ± 0.06	2.55 ± 0.20*

The monoenoic species can be characterized as palmitoyl-palmitoleyl-phosphatidylcholine (16:0/16:1-PC) and palmitoleyl-palmitoyl-phosphatidylcholine (16:1/16:0-PC). Values (%) or absolute content are mean ± S.D., n = 15, *P < 0.05 vs. control

Table 2 shows the relative composition of esterified fatty acids in the phosphatidylcholine of the fetal lungs. The portion of palmitic acid was nearly unchanged in the treated group as compared to the control. The administration of betamethasone decreased the relative proportion of monoenoic fatty acids (16:1, 18:1) and increased that of polyenoic fatty acids (18:2, 20:4) in the esterified fatty acids of pulmonary phosphatidylcholine.

Table 2

Relative composition of esterified phosphatidylcholine fatty acids (%)

	16:0	16:1	18:0	18:1	18:2	20:4
Control (8)	44.1±2.5	9.1±1.4	8.3± 2.6	19.4±2.0	5.4±0.4	6.5±0.5
Betamethasone (9)	45.7±2.2	7.0±0.9*	8.7±1.5	17.0±1.8*	6.4±0.7	8.3±1.1*

Values are mean ± S.D., with the number of animals given in parentheses. *P < 0.05

Comparing the phosphatidylcholine fatty acid profile (Table 3) with changes in the proportion of the 32-carbon-atom monoenoic species in the total phosphatidylcholine species after betamethasone treatment (Table 3), there is a significant percentage decrease of 16:1 in the phosphatidylcholine fatty acids,

which coincides with the reduction in the 16:0/16:1-PC and 16:1/16:0-PC species. In contrast, the reduction of the 18:1 portion in the phosphatidylcholine fatty acids corresponds to the diminution of the 34-carbon phosphatidylcholine portion in the phosphatidylcholine species.

Table 3

Phosphatidylcholine species (% of total phosphatidylcholine)

	PC-32	PC-34	PC-36	PC-38
Control	44.3 ± 6.5	31.7 ± 3.0	13.1 ± 2.0	3.4 ± 0.7
Betamethasone	46.5 ± 7.1	27.4 ± 2.6	13.2 ± 1.7	1.7 ± 0.6*

Values are mean ± S.D. *$P < 0.05$

Anderson et al. (1981) found a marked increase in the uptake of labeled choline in the phosphatidylcholine of fetal lungs as compared to the controls only in the dose range between 0.8 and 6.4 mg/kg betamethasone (administered maternally on the 20th day of gestation). On the other hand, a dose of 0.4 mg dexamethasone has been shown to depress DNA by 51%, total phosphatidylcholine by 28% and disaturated phosphatidylcholine by 33%. In the present study, a decrease in all assayed phosphatidylcholine species as compared to the control values was found after betamethasone had been administered to the pregnant females.

As regards differences in phosphatidylcholine species composition between fetal lungs and the lungs of adult animals, the two monoenoic 32-carbon phosphatidylcholine species (16:0/16:1-PC and 16:1/16:0-PC) are of particular interest. Whereas in the fetal lungs 16:1/16:0-phosphatidylcholine makes up the major portion of the 32-carbon PC monoenoic fraction, in the lungs of adult male rats this phosphatidyl-choline species is only a minor constituent and 16:0/16:1-PC represents the dominant fraction of the 32-carbon PC monoenoic species. There is no notable reversal of the ratio of these two 32-carbon PC monoenoic species following corticoid treatment, as would be expected in the case of an accelerated assimilation of the PC profile of the fetal lung to that of adult animals. On the contrary, both 32-carbon PC monoenoic species undergo a reduction in both absolute and relative terms. The potential importance of these two PC species for the lung fraction or as a predictor of fetal lung maturity has yet to be established. In addition to effecting changes in the PC species composition of fetal lungs and accelerating choline uptake in the pulmonary phosphatidylcholine in both *in vitro* and *in vivo* models, corticosteroids also exert an influence on protein metabolism. A marked reduction in lung DNA content has been reported (Abdellatif and Hollingsworth, 1980; Adamson and Bowden, 1975; Funkhouser and Hughes, 1980), which points to a retardation of pulmonary mitosis, with differentiation of the lung cells being favoured. Thus, morphologic changes in fetal lungs as compared to the

untreated controls have been found 24–48 h after steroid treatment and interpreted as an expression of the functional and morphologic maturation of the lungs (De Lemos et al., 1970; Liggins and Howie, 1972; Picken et al., 1975). In this context, the proliferation of alveolar type II cells (Picken et al., 1975; Smith et al., 1974; Wang et al., 1971), as well as the enhanced formation of lamellated corpuscles in type II cells (Possmayer et al., 1977) are of especial importance. A characteristic feature of type II cells is the high portion of disaturated PC in the PC species (Batenburg et al., 1978; Mason and Williams, 1980). If the number of type II cells increases with respect to the other lung cells, an elevation in the dipalmitoylphosphatidylcholine content should be expected after betamethasone treatment. In addition, a stimulation of PC synthesis in type II cells by steroids has been reported (Adamson and Bowden, 1975; Post et al., 1980; Sanders et al., 1981).

References

Abdellatif, M. M. and Hollingsworth, M. (1980): Effect of oxotremorine and epinephrine on lung surfactant secretion in neonatal rabbits. Pediatr. Res. **14**, 916–921.

Adamson, I. Y. R. and Bowden, D. H. (1975): Reaction of cultured adult and fetal lung to prednisolone and thyroxine. Arch. Pathol. **99**, 80–87.

Anderson, G. G., Lamden, M. P., Cidlowski, J. A. and Ashikaga, T. (1981): Comparative pulmonary surfactant-inducing effect of three corticosteroids in the near-term rat. Obstet. Gynecol. **139,** 562–569.

Bartlett, G. R. (1959): Phosphorus assay in column chromatography. J. Biol. Chem. **234,** 466–468.

Batenburg, J. J., Longmore, W. J. and van Golde, L. M. G. (1978): The synthesis of phosphatidylcholine by adult rat lung alveolar type II epithelial cells in primary culture. Biochim Biophys. Acta **529**, 160–174.

De Lemos, R. A., Shermeta, D. W., Knelson, J. H., Kotas, R. and Avery, M. E. (1970): Acceleration of appearance of pulmonary surfactant in the fetal lamb by administration of corticosteroids. Am. Rev. Resp. Dis. **102**, 459–465.

Dunnett, C. W. (1955): A multiple comparison procedure for comparing several treatments with a control. J. Am. Stat. Assoc. **50**, 1096–1121.

Funkhouser, J. D. and Hughes, E. R. (1980): Fetal lung desaturated phosphatidylcholine. Ostensible increase following exposure to dexamethasone. Biochim. Biophys. Acta **619**, 509–517.

Hamosh, M. and Hamosh, P. (1977): The effect of prolactin on the lecithin content of fetal rabbit lung. J. Clin. Invest. **59**, 1002–1009.

Kanjanapone, V., Hartin-Beecken, I. and Epstein, M. P. (1980): Effect of isoxsuprine on fetal lung surfactant in rabbits. Pediatr. Res. **14**, 278–284.

Liggins, G. C. (1969): Premature delivery of foetal lambs infused with glucococorticoids. J. Endocrinol. **45**, 515–522.

Liggins, G. C. and Howie, R. N. (1972): A controlled trial of antepartum glucocorticoid treatment for prevention of the respiratory distress syndrome in premature infants. Pediatrics **50**, 515–523.

Lipshitz, J., Broyles, K., Hessler, J. R., Whybrew, W. D., Abokas, R. A. and Anderson, G. D. (1981): Effects of hexoprenaline on the L/S ratio and pressure-volume relationship in fetal rabbits. Am J. Obstet. Gynecol. **139**, 726–731.

Lohninger, A. and Nikiforov, A. (1980): Quantitative determination of natural dipalmitoyl lecithin with dimyristoyl lecithin as internal standard by capillary gas-liquid chromatography. J. Chromatogr. **192**, 185–189.

Mason, R. J. and Williams, M. C. (1980): Phospholipid composition and ultrastructure of A549 cells and other cultured pulmonary epithelial cells of presumed type II cell origin. Biochim. Biophys. Acta **617**, 36–45.

Myher, J. J., Kuksis, A., Marai, L., and Yeung, S. K. F. (1978): Microdetermination of molecular species of oligo- and polyunsaturated diacylglycerols by gas-chromatography — mass spectrometry of their tertbutyl dimethylsilyl ethers. Anal. Chem. **50**, 557–564.

Nikiforov, A., Lohninger, A., Specker, M. and Linhart, L. (1982): Studies on molecular species of dog lung sn-glycerol-3-phosphocholines by gas liquid chromatography — mass spectrometry and computer accumulated integrated field desorption mass spectrometry (FD-CAI MS). Eur. J. Mass. Spectrom. Biochem. Med. Environ. Res. **2**, 105–112.

Picken, J. J., Lurie, M. and Kleinerman, J. (1975): Prolonged glucocorticoid treatment in the rat: effects on pulmonary morphology, ultrastructure and elastic properties. Chest **67**, 48S–50S.

Possmayer, F., Duwe, G., Metcalfe, R., Stewart-DeHaan, P. J., Wong, C., Heras, J. L. and Harding, P. G. R. (1977): Cortisol induction of pulmonary maturation in the rabbit foetus: its effects on enzymes related to phospholipid biosynthesis and on marker enzymes for subcellular organelles. Biochem. J. **166**, 485–492.

Possmayer, F., Casola, P. G., Chan, F., MacDonald, P., Ormseth, M. A., Wong, T., Harding, P. G. R. and Tokmakjian, S. (1981): Hormonal induction of pulmonary maturation in the rabbit fetus. Effects of maternal treatment with estadiol-17β on the endogenous levels of choline-phosphate, CDP-choline and phosphatidylcholine. Biochim. Biophys. Acta **664**, 10–22.

Post, M., Batenberg, J. J. and Van Golde, L. M. G. (1980): Effects of cortisol and thyroxine on phosphatidylcholine and phosphatidylglycerol synthesis by adult rat lung alveolar type II cells in primary culture. Biochim. Biophys. Acta **618**, 308–317.

Sanders, R. L., Engle, M. J. and Douglas, W. H. J. (1981): Effect of dexamethasone upon surfactant phosphatidylcholine and phosphatidylglycerol synthesis in organotypic cultures of type II cells. Biochim. Biophys. Acta **664**, 380–391.

Smith, B. T., Torday, J. S. and Giroud, C. J. P. (1974): Evidence for different gestation-dependent effects of cortisol in cultured fetal lung cells. J. Clin. Invest. **53**, 1518–1524.

Sundell, H. W., Gray, M. E., Relier, J. P., Swift, L. L. and Stahlman, M. T. (1979): The effects of ACTH on lung maturation in fetal lambs. Am. J. Pathol. **97**, 393–401.

Wang, N. S., Kotas, R. V., Avery, M. E. and Thurlbeck, W. M. (1971): Accelerated appearance of osmiophilic bodies in fetal lungs following steroid injection. J. Appl. Physiol. **30**, 362–368.

Acta Veterinaria Hungarica 43 (2–3), pp. 303–309 (1995)

PRENATAL STARVATION, BETAMETHASONE AND LUNG DEVELOPMENT IN NEWBORN RATS

S. STETTNER[1] and A. LEDWOŻYW[2]

[1]Ist Clinic of General Surgery, Medical Academy, Jaczewskiego 8, 20–950 Lublin, Poland; [2]Department of Pathophysiology, Agricultural Academy, Lublin, Poland

(Received February 22, 1995)

This study examined the potential benefit of simultaneous transplacental betamethasone, which accelerates fetal lung maturation. Pregnant rats were placed in one of 4 groups: Control (C), fed *ad libitum* until term and given daily physiological saline injections from day 15 of gestation until term; Betamethasone (B), fed as group C but given daily physiological saline injections of 2.0 mg betamethasone/kg body weight from day 15 until term; Starved (S), given 50% rations from day 15 until term and injected as group C; Starved + Betamethasone (SB), fed as group S and injected as group B. Controls and group B did not differ in body or lung weight, protein or DNA, but group B lungs contained more lavageable and tissue surfactant. The S neonates weighed about 40% less than controls, with a proportional reduction in lung weight, DNA, protein or lavage and tissue phospholipids. Betamethasone may alleviate the impact of starvation on the developing lung by accelerating the process of alveolarization which was solved by caloric deprivation.

Key words: Rat, starvation, glucocorticoids, lung development

Prenatal caloric restriction (starvation) retards fetal lung development and is associated with an increased number of stillbirths and "frail-to-thrive" neonates that die within hours or days of birth (Lechner, 1984). In guinea pigs that are normally born with functionally mature lungs (Lechner and Banchero, 1982), starvation in the last trimester of pregnancy causes pulmonary hypoplasia, decreased anatomical diffusing capacity, altered tissue elastic recoil, and reduced surfactant production and release (Lechner et al, 1986). Similarities in lung structure and function between newborn rats after prenatal starvation and human premature or growth-retarded infants (Langston et al., 1984; Lechner and Tull, 1986; Morrison and Olsen, 1985) suggest that these animal studies are useful models of clinical conditions such as prematurity and its postnatal consequences.

Glucocorticoids are currently used for reducing the incidence of respiratory distress syndrome (RDS) in premature and low-birthweight infants. Works with betamethasone and dexamethasone have shown that glucocorticoids regulate or enhance proliferation and differentiation of squamous type I cells, maturation of type II cells via fibroblasts, airspace enlargement and distensibility, septal thin-

0236–6290/95/$ 4.00 © 1995 *Akadémiai Kiadó, Budapest*

ning, capillary infiltration and mesenchymal remodeling (Beer et al., 1984; Mitzner et al., 1982; Post et al., 1986). These compounds also stimulate the production or appearance of adrenergic and muscarinic receptors and prostaglandins in the fetal lung, which may thereby regulate airway reactivity, vascular resistance and surfactant release (Marquardt et al., 1982; Tsai et al., 1984). Despite disappointing initial clinical trials using glucocorticoids to prevent RDS (Collaborative Group, 1981), additional surveys have substantiated their effectiveness, with no significant postnatal growth retardation or drug-related pathologies (Doyle et al., 1976; Morales et al., 1986).

Because the normal human lung is not prevasively saccular at birth like that of the rat, the starved rat model is particularly relevant to situations of intrauterine growth retardation in humans. The selection of drug dosage and duration for these experiments was made so as to optimize biochemical changes in the fetal lungs (Nelson et al., 1976). The present results substantiate the concept that glucocorticoids exhibit clinically beneficial effects by altering lung development in multiple ways.

Materials and methods

Pregnant Wistar rats with verified dates of insemination were individually caged at 22 °C, under a 12:12 h light:dark cycle, and 40 to 70% relative humidity in an isolation room. All were fed *ad libitum* (LSM chow, 18.5% protein, 4.0% fat, 11.5% fibre until day 15 post insemination when they were randomly assigned to one of 4 groups. Controls (C) continued to receive chow *ad libitum* until term and were injected with 0.5 ml sterile 0.9% saline intramuscularly from day 15 of gestation until term. Betamethasone animals (B) were fed as group C, but were injected from day 15 with 2.0 mg betamethasone (Betapred, Glaxo, UK, 4 mg/ml) per kg maternal body weight. Starved females (S) were given 50% rations (about 22 g chow/day) from day 15 until term, and injected with saline as group C. Animals that were both starved and treated with betamethasone (SB) were fed as group S and injected as group B. Water was provided *ad libitum* and supplemented with 50 mg/l of vitamin C during starvation. The dosage and timing of betamethasone injections were interpolated from available literature for this and other species covering a range of weights and lengths of gestation (Kaufmann, 1971; Mitzner et al., 1982; Nelson et al., 1976; Sanfacon et al., 1977; Tsai et al., 1984).

Neonates were removed from their mothers within 6 h of birth weighed, and their sex was determined. They were designated as healhty and suitable for further analyses if they were active and nursing, had rectal temperatures of at least 33 °C, and were not shivering or hyperventilating at room temperature. Other liveborn neonates were classified as "fail-to-thrive"if they were hypothermic, cyanotic,

shivering or were hyperventilating excessively. Monitoring of such animals continued until their inevitable death within the next 3 to 36 h. Stillborn animals were blotted dry, weighed, and their sex was determined.

Some newborn rats were randomly selected from each group for biochemical analyses. They were anaesthetized (sodium pentobarbital, 30 mg/kg, i. p.) and their body lengths (snout to anus) measured.

After tracheotomy and intubation with a rigid Luer stub adapter (18–21) gauge, the lungs were collapsed by puncture of the diaphragm and lavaged *in situ* 5 times with sterile 0.9% NaCl, with each volume equal to the estimated vital capacity at 20 cm H_2O. Following lavage, the lungs and hearts were quickly excised *en bloc,* separated from the primary bronchi and great vessels, respectively, and then rinsed in saline, blotted and weighed. The lavaged and separated lungs were frozen at -20 °C until further analysis.

Lavage volumes were determined and then the samples centrifuged at 200 *g* for 10 min to remove alveolar macrophages and debris. A 2.5 ml aliquot of each was extracted in chloroform-methanol (1:1) and NaCl solutions according to Folch et al. (1957). The right lung, exclusive of the large medial lobe, was homogenized in 20 times its lavaged weight of chloroform-methanol (2:1), extracted and filtered (Folch et al., 1957). Both lavage and tissue extracts were dried under N_2, resuspended in chloroform and assayed for total phosphorus (Bartlett, 1959) and for constituent phospholipids using two-dimensional thin-layer chromatography (silica gel G precoated plates, E. Merck, Darmstadt, Germany) (Lechner et al., 1986). The entire left frozen lung was homogenized in cold saline and assayed for total DNA and nonblood proteins using diphenylamine reaction and microbiuret procedure with haemoglobin correction, respectively (Lechner, 1985*a*; Lechner et al., 1986). The frozen right medial lobe was lyophilized to constant dry weight. Total lung dry weight, DNA content, protein content, and phospholipid concentrations were then computed.

Data are presented as mean ± S. D. with significance among the experimental groups tested by analysis of variance (ANOVA) and the Student Neuman-Keul *a posteriori* test, and frequency data tested using the chi-square distribution (Sokal and Rohlf, 1969).

Table 1

Body morphometrics in newborn guinea pigs

	C	B	S	SB
Body weight, g	44 ± 2	44 ± 2	3w ± 2*	33 ± 2*
Body length, mm	33 ± 2	32 ± 2	25 ± 2*	24 ± 2*

Values are mean ± S.D.; n = 17; *P < 0.04 vs. control. Group designation as in Materials and methods

Results

Based on the length and weight indices presented in Table 1, prenatal starvation resulted in proportionally smaller neonates, characterized as nutritional dwarfs (Lechner, 1984; Lechner, 1985*b*). None of these parameters was significantly affected by one treatment.

Table 2

Total dry lung weight, DNA and nonblood protein for newborn rats

	C	B	S	S + B
Dry lung weight, mg	155 ± 11	160 ± 14	61 ± 7*	59 ± 6*
DNA, mg	9 ± 1	8 ± 1	5 ± 1*	4 ± 1*
Nonblood protein, mg	97 ± 7	100 ± 8	65 ± 5*	70 ± 7*

Values are mean ± S.D.; n = 17; *P < 0.04 vs. control. Group designation as in Materials and methods

Table 3

Surfactant phospholipids isolated from newborn rats

	C	B	S	S + B
Lavage phospholipids				
Total phosphorus, μg	222 ± 18	430 ± 45*	158 ± 18*	156 ± 18*
DSPC, %	57 ± 5	56 ± 4	54 ± 3	49 ± 5
USPC, %	12 ± 2	11 ± 1	10 ± 2	10 ± 2
PG, %	8 ± 1	8 ± 1	8 ± 1	9 ± 2
Tissue phospholipids				
Total phosphorus, μg	1555 ± 100	1880 ± 120*	1140 ± 70*	1100 ± 80*
DSPC, %	28 ± 3	28 ± 4	26 ± 3	27 ± 3
USPC, %	14 ± 1	12 ± 2	15 ± 2	13 ± 2
PG, %	17 ± 2	19 ± 2	18 ± 2	17 ± 1
PI, %	8 ± 1	8 ± 1	9 ± 1	8 ± 1

Values are mean ± S.D.; n = 17; *P < 0.04 vs. control. DSPC: disaturated phosphatidylcholine; USPC: unsaturated phosphatidylcholine; PG: phosphatidylglycerol; PI: phosphatidylinositol. Group designation as in Materials and methods

Prenetal starvation caused significant reductions in lung dry weight, DNA and nonblood protein in group S compared with controls ($p < 0.01$ for all, Table 2). Reductions of this magnitude were also found among the SB neonates, whereas newborn rats from well-fed, betamethasone treated litters (group B) did not differ from controls in these parameters. However, group B animals did show significant

elevations in both lavage and tissue phospholipids over control levels. Prenatal starvation (groups S and SB) resulted in significant phospholipid reductions in both lung lavage and tissue, regardless of the presence of prenatal betamethasone.

There were no significant changes or trends in surfactant composition, with disaturated phosphatidylcholine being the major lavage and tissue phospholipid in all 4 groups (Table 3).

Discussion

Prenatal betamethasone influenced survival among newborn rats regardless of the maternal feeding regimen. Although the overall mean body weights for all term fetuses were not altered by betamethasone, drug treatment led to higher frequencies of healthy neonates and lower average body weights of sickly and stillborn animals. These data substantiated the subjective impression of improved vigour in the smallest SB neonates compared with their starved, saline-injected counterparts. Similar findings have been reported in very low birthweight infants whose mothers received prenatal glucocorticoids. Those infants required less assisted ventilation at lower F_{102} and shorter hospitalizations than controls, while showing a lower incidence of intraventricular haemorrhage and no significant side effects such as increased infections, altered adrenal activity, or permanent growth retardation (Dorr et al., 1986; Doyle et al., 1986; Morales et al., 1986).

Glucocorticoids appear to improve neonatal survival after premature delivery or following intrauterine growth retardation by accelerating fetal lung maturation, as typified by the data for group B animals. In the dosage range reported here, tissue and lavageable phospholipids increase markedly (Collaborative Group, 1981; Hitchcock, 1980; Kotas and Avery, 1971; Nelson et al., 1976). Increased phospholipids were in turn thought to be responsible for the greater maximal V_L and improved deflation stability of lungs from glucocorticoid-treated animals (Hitchcock, 1980; Post et al., 1986).

It cannot be determined at present whether these improvements could by themselves account for the improved viability noted, although the fetal rat lung is clearly responsive to betamethasone in both starved and well-fed litters. Receptors for glucocorticoids have been localized in fetal mouse lung within both mesenchymal and distal epithelial cells (Beer et al., 1984; Hitchcock, 1980) where they may mediate production of extracellular matrix proteins as well as growth factors like fibroblast pneumocyte factor (Post et al., 1986). Mulay et al. (1982) found no loss of cytoplasmic glucocorticoid receptors in fetal rat lungs following a low-protein diet throughout gestation, but they speculated that altered nuclear binding could explain the reduced surfactant production reported by others (Faridy, 1975). Starvation and fasting have also been reported to cause down-regulation of beta adrenergic receptors in other tissues (Weick and Ritter, 1983;

Young et al., 1978), but this phenomenon has not been systematically examined in prenatal malnutrition. The intriguing possibility exists that some of the pulmonary deficiencies reported here for the S group neonates and in the starved fetuses documented by others (Curle and Adamson, 1978; Faridy, 1975; Naeye et al., 1974) are the result of a similar loss of responsiveness to circulating catecholamines. Loss of beta-receptor numbers or affinity could drastically reduce surfactant release, lung water reabsorption, airway and vascular tone, and pulmonary compliance (Bergman, 1981; Vilos et al., 1985). Betamethasone and other gucocorticoids enhance beta-receptor density and function in a variety of organ systems (Davies and Lefkowitz, 1984; Salonen, 1985; Salonen and Mattila, 1984).

This indirect action of glucocorticoids in regulating early lung development via adrenergic receptors has been verified through the use of propranolol, which blocked the normal acceleration of fetal lung maturation by adrenocorticotropin (Vilos et al., 1985). Thus, prenatal betamethasone could improve survival and accelerate lung maturity in part by reversing adrenergic down-regulation induced by caloric restriction.

References

Bartlett, G. R. (1959): Phosphorus assay in column chromatography. J. Biol. Chem. **234**, 466–468.

Beer, D. G., Butley, M. S., Cunha, G. R. and Malkinson, A. M. (1984): Autoradiographic localization of specific [^{3}H] dexamethasone binding in fetal lung. Develop. Biol. **105**, 351–364.

Bergman, B. (1981): Beta-mimetics and the preterm neonatal lung. Acta Physiol. Scand. (Suppl. **497**), 179–186.

Collaborative Group on Antenatal Steroid Therapy (1981): Effect of antenatal dexamethasone administration on the prevention of respiratory distress syndrome. Am. J. Obstet. Gynecol. **141**, 276–286, 1981.

Curle, D. C. and Adamson, I. Y. R. (1978): Retarded development of neonatal rat lung by maternal malnutrition. J. Histochem. Cytochem. **26**, 401–408.

Davies, A. O. and Lefkowitz, R. J. (1984): Regulation of β-adrenergic receptors by steroid hormones. Annu. Rev. Physiol. 46, 119–130.

Dorr, H. G., Versmold, H. T., Sippel, W. G., Bildingmaier, F. and Knorr, D. (1986): Antenatal betamethasone therapy: effects on maternal, fetal and neonatal mineralocorticoids, glucocorticoids and progestins. J. Pediatr. **108**, 990–993.

Doyle, L. W., Kitchen, W. H., Ford, G. W., Rickards, A. L., Lissenden, J. V. and Ryan, M. M. (1986): Effect of antenatal steroid therapy on mortality and morbidity in very low birth weight infants. J. Pediatr. **108**, 287–292.

Faridy, E. E. (1975): Effect of maternal malnutrition on surface activity of fetal lungs in rats. J. Appl. Physiol. **39**, 535–540.

Folch, J., Lees, M. and Sloane-Stanley, G. H. (1957): A simple method for the isolation and purification of total lipids from animal tissues. J. Biol. Chem. **226**, 497–509.

Hitchcock, K. R. (1980): Lung development and the pulmonary surfactant system: hormonal influences. Anat. Rec. **198**, 13–34.

Kaufmann S. L. (1971): Proliferation, growth and differentiation of pulmonary epithelium in fetal mouse lung exposed transplacentally to dexamethasone. Lab. Invest. **37**, 497–501.

Kotas, R. V. and Avery, M. E. (1971): Accelerated appearance of pulmonary surfactant in the fetal rabbit. J. Appl. Physiol. **30**, 358–361.

Langston, C., Kida, K., Reed, M. and Thurlbeck, W. M. (1984): Human lung growth in late gestation and in the neonate. Am. Rev. Resp. Dis. **129**, 607–613.

Lechner, A. J. (1984): Growth retardation and mortality in guinea pigs following perinatal starvation. Nutr. Rep. Int. **30**, 1435–1447.

Lechner, A. J. (1985*a*): Starvation-induced organ hypoplasia in prenatal and postnatal guinea pigs. Biol. Neonate **48**, 36–42.

Lechner A. J. (1985*b*): Perinatal age determines the severity of retarded lung development induced by starvation. Am. Rev. Resp. Dis. **131**, 638–643.

Lechner, A. J. and Banchero, N. (1982): Advanced pulmonary development in newborn guinea pigs (*Cavia porcellus*). Am. J. Anat. **163**, 235–246.

Lechner, A. J. and Tull, D. S. (1986): Prenatal starvation retards development of the ventillatory response to hypoxia in newborn guinea pigs. Pediatr. Res. **20**, 920–924.

Lechner, A. J., Winston, D. C. and Bauman, J. E. (1986): Lung mechanics, cellularity and surfactant after prenatal starvation in guinea pigs. J. Appl. Physiol. **60**, 1610–1614.

Marquardt, D. L., Motulski, H. J. and Wasserman, S. I. (1982): Rat lung cholinergic receptor: characterization and regulation by corticosteroids. J. Appl. Physiol. **53**, 731–736.

Mitzner, W., Johnson, J. W. C., Beck, J., London, W. and Sly, D. (1982): Influence of betamethasone on the development of mechanical properties in the fetal rhesus monkey lung. Am. Rev. Resp. Dis. **125**, 233–238.

Morales, W. J., Diebel, N. D., Lazar, A. J. and Zadrozny, D. (1986): The effect of antenatal dexamethasone administration on the prevention of respiratory distress syndrome in preterm gestation with premature rupture of membranes. Am. J. Obstet. Gynecol. **154**, 591–595.

Morrison, I. and Olsen, J. (1985): Weight-specific stillbirth and associated causes of death: an analysis of 765 stillbirths. Am. J. Obstet. Gynecol. **152**, 975–980.

Mulay, S., Varma, D. R. and Solomon, S. (1982): Influence of protein deficiency in rats on hormonal status and cytoplasmic glucocorticoid receptors in maternal and fetal tissues. J. Endocrinol. **95**, 49–58.

Naeye, R. L., Freeman, R. K. and Blanc, W. A. (1974): Nutrition, sex and fetal lung maturation. Pediatr. Res. **8**, 200–204.

Nelson, G. H., Eguchi, K. and McPherson, J. C. (1976): Effects of gestational age, dexamethasone and metopirone on lecithin concentration in fetal lung tissue and amniotic fluid in rats and guinea pigs. Gynecol. Invest. **7**, 337–343.

Post, M., Barsoumian, A. and Smith, B. T. (1986): The cellular mechanism of glucocorticoid acceleration of fetal lung maturation. J. Biol. Chem. **261**, 2179–2184.

Salonen, R. O. (1985): Concomitant glucocorticoid treatment prevents the development of desensitization in the guinea pig lung. Acta Pharmacol. Toxicol. **57**, 147–153.

Salonen, R. O. and Mattila, M. J. (1984): A 24-hour glucocorticoid treatment increases the number of adrenoceptors in the rat lung. Acta Pharmacol. Toxicol. **55**, 425–428.

Sanfacon, R., Possmayer, F. and Harding, P. G. R. (1977): Dexamethasone treatment of the guinea pig fetus: its effects on the incorporation of ^{3}H-thymidine into deoxyribonucleic acid. Am. J. Obstet. Gynecol. **127**, 745–752.

Sokal, R. R. and Rohlf, R. J. (1969): Biometry. 1st ed. W. H. Freeman, San Francisco.

Tsai, M. Y., Handschin, B., Syverson, B. J., Josephson, M. W. and Brown, D. M . (1984): Effect of prenatal dexamethasone on immunoreactive 6-keto prostaglandin $F_{1\alpha}$ levels in fetal rat lungs. Pediatr. Res. **18**, 908–911.

Vilos, G. A., Challis, J. R. G., Pliagas, G. A., Lye, S. L., Possmayer, F. and Harding, P. G. (1985): Propranolol inhibits the maturational effect for adrenocorticotropin in the fetal sheep lung. Am. J. Obstet. Gynecol. **153**, 472–477.

Weick, B. G. and Ritter, S. (1983): Fasting reduces cardiac beta-adrenergic receptor concentration and ornithine decarboxylase activity in rats. J. Auton. Nerv. Syst. **7**, 179–193.

Young, J. B., Mullen, D. and Landsberg L. (1978): Caloric restriction lowers blood pressure in the spontaneously hypertensive rat. Metabolism **27**, 1711–1715.

Acta Veterinaria Hungarica 43 (2–3), pp. 311–320 (1995)

PROTECTIVE EFFECT OF VERAPAMIL ON REGIONAL MYOCARDIAL ISCHAEMIC INJURY IN DOG

W. KOBUSIEWICZ[1], Maria OGONOWSKA-KOBUSIEWICZ[2], J. MICHALAK[1], W. ŻYWICKI[1] and A. LEDWOŻYW[3]

[1]Clinic of Vascular Surgery and [2]Department of Anaesthesiology, Medical Academy, Jaczewskiego 8, 20–090 Lublin, Poland; [3]Department of Pathophysiology, Agricultural Academy, Lublin, Poland

(Received January 5, 1995)

Ischaemic injury was produced in the dog heart by occluding the left anterior descending coronary artery just below the second diagonal branch for 3 h. Verapamil 5.0 mg/kg was injected intravenously 10 min before the occlusion. There was some decrease in ATP and creatine phosphate levels in the hearts of "no drug" animals as compared to sham-operated dogs. Verapamil significantly increase the level of these metabolites. The ATP/ADP ratio decreased from 8.5 ± 0.60 in hearts from sham-operated dogs to 3.5 ± 0.30 in the "no drug" group. Verapamil pretreatment resulted in values of the ATP/ADP ratio which were lower than, but not significantly different from, those observed in the control group. In ischaemic mitochondria the calcium content (Ca^{++}) increased significantly as compared to the control and the Verapamil group. A significant increase in free fatty acid content in myocardial membrane preparations was also observed as compared to the sham-operated and the Verapamil group.

Key words: Heart, ischaemia, mitochondria, calcium, free fatty acids, creatine phosphate, adenine nucleotides

Several calcium slow channel blocking agents have been shown to reduce damage to hearts due to experimental ischaemia. The mechanisms of preservation of ischaemic hearts by these treatments are not yet clear, but include both vascular and myocardial components. Slow channel blockers can increase coronary artery collateral flow and decrease the afterload by dilating peripheral vessels. These agents can reduce myocardial oxygen consumption by decreasing contractility (Jolly and Gross, 1979), thus preserving energy reserves during ischaemia (Ross-Nicholson et al., 1978; Watts et al., 1980). Slow channel blockers also decrease the calcium overload upon reperfusion (Bourdillon and Poole-Wilson, 1982; Higgins and Blackburn, 1984) and may have intracellular sites of action in addition to those induced by interaction with the plasma membrane (Janis and Scriabine, 1983; Janis and Triggle, 1983).

0236–6290/95/$ 4.00 © 1995 *Akadémiai Kiadó, Budapest*

Watts et al. (1985) have demonstrated that the protection of globally ischaemic rat hearts by verapamil is in part associated with energy preservation, but additional mechanisms may also be involved.

The protective effect of slow channel blocking agents against myocardial ischaemic injury has been observed with verapamil (Ross-Nicholson et al., 1978), diltiazem (Bush et al., 1981; Ichihara and Abiko, 1983) and nifedipine (Clark et al., 1977; Henry et al., 1979).

This study presents the effect of verapamil on adenine nucleotides and creatine phosphate in the partially ischaemic dog heart.

Recent advances have revealed that a decrease in membrane phospholipids, which are the major constituents of biomembrane, plays an important role in the development of various phathological conditions (Katz and Messineo, 1981).

Miyazaki et al. (1984) have observed an increase in mitochondrial free fatty acids (FFA) in association with the appearance of reperfusion arrhythmia and suggested that the activation of phospholipase (PLase) resulting in decrease of membrane phospholipids associated with coronary reperfusion is closely related to the appearance of reperfusion responses.

This study is designed also to clarify the mechanism of ischaemic heart arrhythmias in relation to the role of PLase and the alterations of plasma membrane phospholipids and FFA, including the effect of Verapamil, a calcium channel blocker.

Materials and methods

The experiment was performed on 25 male mongrel dogs (bodyweight range 18.5–25.5 kg). The dogs were divided into 3 groups: (1) sham-operated (thoracotomy and pericardiotomy only; n = 9), (2) control ("no drug"; n = 8); (3) Verapamil-supplemented animals (5 mg/kg; n = 8). The drug was administered just before the coronary artery occlusion.

Animals were anaesthetized with i. v. infusion of sodium pentobarbital (30 mg/kg) and Fentanyl (0.05 mg/kg). Surgical anaesthesia was maintained after endotracheal intubation with $N_2O + O_2$ 2:1 v/v. Supplemental doses of Fentanyl (0.05 mg/kg) were administered if and when needed to abolish the corneal reflex. Positive pressure was maintained at 16 cycles/min, and tidal volume of 15 ml/kg body weight using a Chirana respirator. Pancuronium (0.1 mg/kg, i.v.) was administered to prevent reflex muscular movement. A teflon cannula with 3-way stopcock was inserted into the left femoral artery for drug administration. The heart was exposed under sterile conditions through a midsternotomy, and after pericardiotomy, the left anterior descending coronary artery was ligated approx. 15–30 mm distal to the tip of the left atrial appendage. In each case complete occlusion was evidenced by the appearance of a nonpulsatile, nondistended artery,

distal to the occlusion and nondistended veins draining from the ischaemic zone, and by the immediate appearance of cyanosis and the bulging of the ischaemic segment. A bolus of 20 mg xylocain was administered i. v. to suppress arrhythmias. At 3 h following the occlusion, the experiment was terminated, the heart was removed, the ligature cut and the heart perfused with cold (4 °C) 0.02 M Tris-HCl buffer, pH 7.4. Samples were taken from the center of the visible ischaemic area in sham-operated dogs, and transmural samples from the area parallel to necrotic zone in control and drug-supplemented dogs.

Myocardial plasma membrane was prepared by the method of Williams et al. (1977). Heart mitochondria were prepared by the method of Hatefi et al. (1961).

Measurement of plasma membrane phospholipids

Plasma membrane lipids were extracted by the method of Folch et al. (1957). Two ml of membrane suspension (20 mg protein) were extracted with 25 ml of a $CHCL_3$/methanol mixture (2 : 1 by vol.). After filtering through a filter paper, 4 ml of 0.73% aqueous NaCl solution were added, mixed vigorously and then centrifuged. The lower phase containing essentially all of the membrane lipids was taken and evaporated to dryness with a rotary evaporator. The content of phospholipids in the membrane was determined by measuring the phosphorus content according to Allen (1940).

Measurement of plasma membrane fatty acids

FFA in plasma membrane (10 mg protein) were extracted with a $CHCL_3$/methanol (2 : 1) solution and evaporated to dryness as described in the previous section. The dried extract was dissolved in 2.0 ml of methanol. To 0.5 ml of the resulting solution 58 nmoles of heptadecanoic acid were added as an internal standard, fatty acids were converted to the corresponding methyl esters with BF_3-methanol and analyzed by a gas chromatographic method as described previosly (Michalak et al., 1988).

Determination of the calcium content of mitochondria

Ca^{++} content of mitochondria was measured by an atomic absorption spectrophotometer.

Effect of phospholipases on FFA content in myocardial membrane

Myocardial plasma membrane samples were divided into 3 groups of 10 mg protein each: no addition of PLase, PLase A_2 1 unit and PLase C 2 units. Immediately after incubation for 5 min at 37 °C, FFA were extracted and analyzed as described above.

The portions of hearts from the ischaemic region were frozen and crushed with dry ice. Cooled mortar and pestle and neutralized perchloric acid extracts were assayed by standard enzymatic procedures for adenine nucleotides (Williamson and Corkey, 1969) and for creatine phosphate (Lamprecht et al., 1974).

Statistical analysis

Analysis of variance tests were performed to determine if treatment effects were significant and the Tukey-Kramer test was used to test individual treatment effects (Sokal and Rohlf, 1981).

Results

There was some decrease in ATP and creatine phosphate levels in the hearts of "no drug" animals as compared to sham-operated dogs. Verapamil significantly increased the level of these metabolites (Fig. 1).

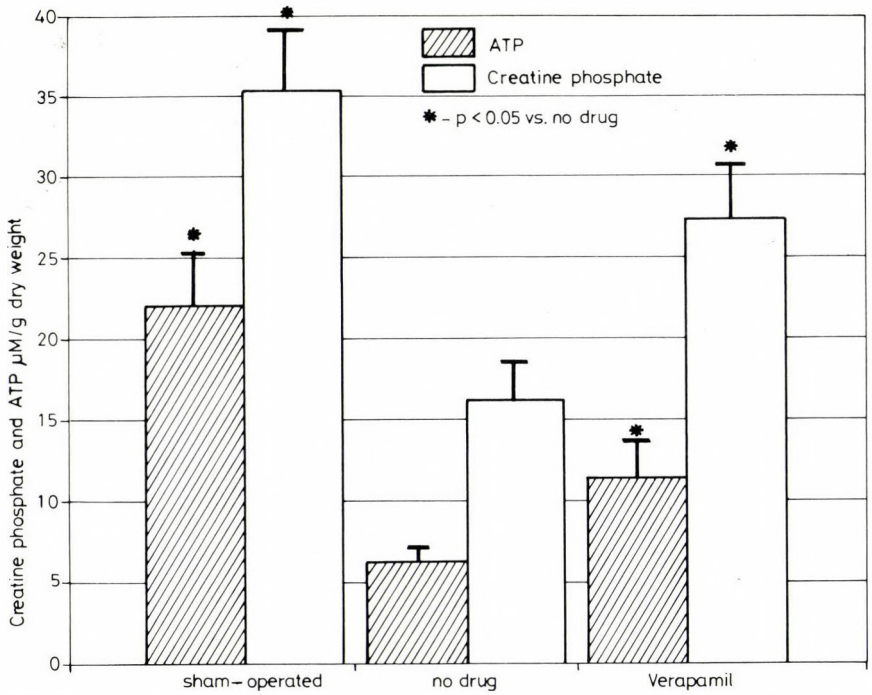


Fig. 1. High-energy phosphates in dog myocardium (mean ± S.D.)

The ATP/ADP ratio decreased from 8.5 ± 0.60 in hearts from sham-operated dogs to 3.5 ± 0.30 in the "no drug" group. Verapamil pretreatment resulted in

values of the ATP/ADP ratio which were lower than, but not significantly different from, those observed in control group (Fig. 2).

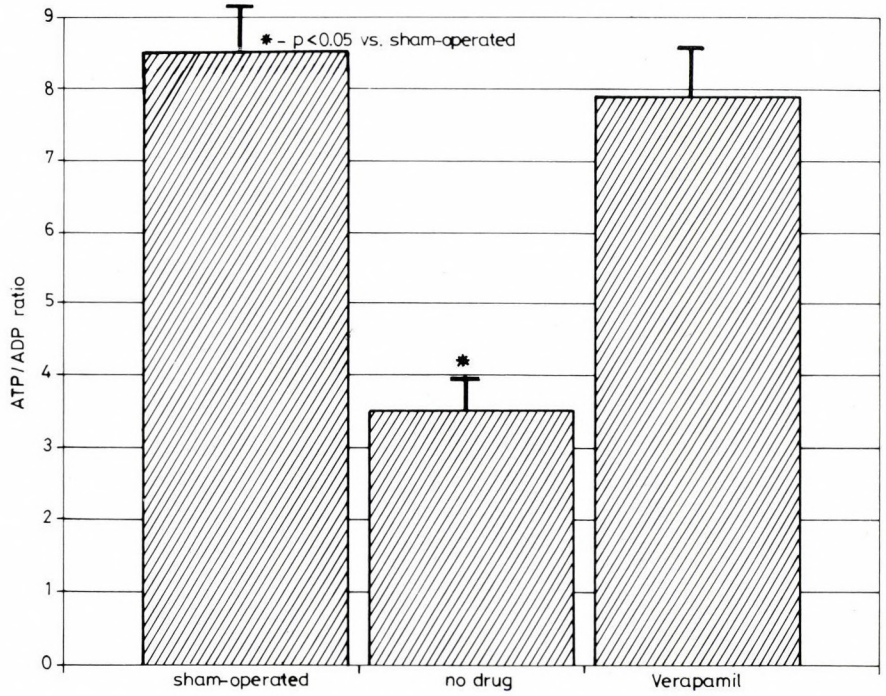


Fig. 2. ATP/ADP ratio in ischaemic heart (mean ± S.D.)

Table 1 shows the content of phospholipids in sham-operated, "no drug" and Verapamil groups. In the "no drug" group the phospholipid content of the membrane preparation decreased significantly as compared with that in the membrane preparation from the hearts of sham-operated dogs. In the Verapamil group no significant difference was observed in the phospholipid content as compared to sham-operated animals.

Table 2 shows the FFA content of the membrane preparation in the sham-operated, "no drug", and Verapamil groups. In the "no drug" group a significant increase in FFA content of the myocardial membrane preparation was observed as compared to the sham-operated and Verapamil group.

Table 3 shows the calcium content of mitochondria. In the "no drug" group Ca^{++} increased significantly in the mitochondria as compared to the control and the Verapamil group.

Table 4 presents the FFA composition of the myocardial plasma membrane preparation. Incubation of membrane samples with PLase A_2 and PLase C induced an increase in the total FFA amount. PLase A_2 induced an increase only in

the unsaturated FFA, without increasing any of the saturated ones. PLase C indeced a significant increase in all of the detected FFA.

Table 1

Content of phospholipids in myocardial membrane (μM/mg protein)

	Phospholipids
Sham-operated	0.32 ± 0.05
No drug	0.22 ± 0.02*
Verapamil	0.30 ± 0.04

Mean ± S.D. *P < 0.05 vs. sham-operated

Table 2

Content of free fatty acids in myocardial membrane (μM/mg protein)

Fatty acid	Sham-operated	No drug	Verapamil
12:0	0.03 ± 0.01	0.08 ± 0.03*	0.04 ± 0.02
14:0	0.36 ± 0.06	0.64 ± 0.05*	0.34 ± 0.05
16:0	1.45 ± 0.20	2.24 ± 0.19*	1.50 ± 0.16
16:1	0.06 ± 0.02	0.20 ± 0.03*	0.04 ± 0.02
18:0	0.66 ± 0.04	1.36 ± 0.15*	0.69 ± 0.05
18:1	0.80 ± 0.10	1.25 ± 0.10*	0.83 ± 0.06
18:2	0.85 ± 0.05	1.36 ± 0.08*	0.82 ± 0.06
20:4	0.40 ± 0.05	0.82 ± 0.06*	0.43 ± 0.04
22:6	0.33 ± 0.03	0.45 ± 0.04*	0.31 ± 0.03

Mean ± S.D. *P < 0.05 vs. sham-operated

Table 3

Ca^{++} content in mitochondria (μM/mg protein)

	Ca^{++} content
Sham-operated	9.5 ± 1.9
No drug	16.6 ± 2.2*
Verapamil	9.8 ± 1.8

Mean ± S.D. *P < 0.05 vs. sham-operated

Table 4

Free fatty acids in myocardial membrane after phopholipase treatment (μM/mg protein)

Fatty acid	Control	PLase A_2	PLase C
12:0	0.05 ± 0.02	0.06 ± 0.02	0.12 ± 0.03*
14:0	0.34 ± 0.08	0.32 ± 0.09	0.50 ± 0.04*
16:0	1.60 ± 0.14	1.64 ± 0.12	1.97 ± 0.13*
16:1	0.08 ± 0.02	0.85 ± 0.06*	0.17 ± 0.02*
18:0	0.66 ± 0.05	0.68 ± 0.06	1.09 ± 0.08*
18:1	0.85 ± 0.10	5.78 ± 0.66*	1.35 ± 0.12*
18:2	0.78 ± 0.09	7.32 ± 0.94*	1.25 ± 0.11*
20:4	0.45 ± 0.03	1.95 ± 0.22*	0.88 ± 0.06*
22:6	0.30 ± 0.02	1.95 ± 0.15*	0.65 ± 0.04*

Mean ± S.D. *$P < 0.05$ vs. control

Discussion

In the present study an evidence is presented that Verapamil protects the heart from partial ischaemic injury. Protection was assessed by the maintenance of increased levels of high energy phosphates. Pretreatment of animals with Verapamil prior to ischaemia provided markedly enhanced high energy stores after cardiac ischaemia, in comparison to those values in non-ischaemic hearts.

There are several potential mechanisms which could explain the direct preservation of the myocardium by Ca^{++} slow channel blockers during ischaemia. These mechanisms might include preservation of ATP by decreased contractility, preservation of this compound without decreased contractility and the protection of cellular processes other than the maintenance of high energy phosphates.

Several authors have demonstrated an association between the treatment of hearts or myocytes with Ca^{++} slow channel blockers and protection of ATP stores following ischaemia DeJong et al. (1982) with nifedipine in isolated, Langendorff perfused rat hearts, Nayler et al. (1980) and Nayler (1982) with nifedipine and Verapamil in isolated, Langendorff perfused rabbit hearts, Weishaar et al. (1979) with diltiazem in the intact dog, and Cheung et al. (1984) with Verapamil and nifedipine in isolated rat myocytes under anoxic conditions.

The mechanism of this preservation of ATP may be a decrease in energy utilization during ischaemia which would reduce the depletion of energy stores and/or a protection of mitochondrial function so that the hearts would be better equipped for functioning. It is accepted that the re-establishment of normal

creatine phosphate levels after ischaemia implies functional energy-producing pathways (Reibel and Rovetto, 1978).

The present results show that creatine phosphate levels are significantly lower in ischaemic hearts which had no drug pretreatment, but are similar in ischaemic hearts in the Verapamil-pretreated group. In addition, the ATP/ADP ratio was higher in the treated than in the untreated hearts. Perhaps the untreated ischaemic hearts, therefore, suffered mitochondrial damage and the hearts of dogs which received Verapamil pretreatment before ischaemia benefited from a protection of the mitochondrial energy-producing mechanisms.

Several observations suggest that energy preservation is not the only mechanism for the action of Ca^{++} antagonists on ischaemic hearts. Some authors were unable to detect an effect of Ca^{++} antagonism on ATP levels in the ischaemic heart (Lamprecht et al., 1974). Watts et al. (1985) have shown that the enhanced recovery of cardiac function observed in globally ischaemic rat hearts was dependent upon the concentration of Verapamil, while the increase in tissue ATP, creatine phosphate or adenine nucleotide pool was not concentration dependent. Neely and Grotyohann (1984) have shown a dissociation of ATP levels and recovery of function in reperfused globally ischaemic rat hearts. They postulate a role of glycolytic products in ischaemic injury. It is possible that the Ca^{++} slow channel blockers also act upon other subcellular processes.

Therefore there are sets of data concerning the existence of contractile-dependent and contractile-independent as well as energy-dependent and energy-independent mechanisms of direct myocardial protection by these agents.

Further studies comparing the effects of various Ca^{++} slow channel blockers within one model of ischaemia should indicate similarities or differences in their mechanisms of action in ischaemic hearts.

In this study we have observed a decrease in phospholipids and an increase in FFA in plasma membrane samples from ischaemic hearts, suggesting that the degradation of membrane phospholipids was caused by the activation of PLase. Although the PLases play important physiological roles (Lands, 1960; Van Den Bosh, 1980), enhanced activation of PLases could degrade membrane phospholipids, resulting in the disturbance of cellular function.

It has been reported (Janis and Triggle, 1983; Jolly and Gross, 1979) that Ca^{++} is an essential factor in activating PLases. The Ca^{++} influx is known to increase in the cells of ischaemic heart. In the present study we also confirmed a significant increase in the Ca^{++} content in ischaemic mitochondria. Williamson et al. (1976) suggested that the accelerated rate of Ca^{++} uptake by ischaemic mitochondria is a reflection of an increased intracellular Ca^{++} concentration since a rise of extramitochondrial Ca^{++} concentration is the major factor that determines energy-linked Ca^{++} uptake by mitochondria.

Verapamil, a member of the group of drugs termed calcium antagonists, is a potent inhibitor of calcium influx through plasma membranes. In this study, the

premedication with Verapamil effectively prevented the increase in Ca^{++} content in ischaemic mitochondria with alterations of membrane phospholipids and FFA. These results lead us to believe that the protective effect of Verapamil is, at least in part, based on its interference with calcium influx into myocardial cells following ischaemia and that this inhibition protects the digestion of membrane phospholipids by phospholipases.

References

Allen, R. J. L. (1940): The estimation of phosphorus. Biochem J. **34B**, 858–861.

Bourdillon, P. D. and Poole-Wilson, P. A. (1982): The effects of verapamil, quiescence and cardioplegia on calcium exchange and mechanical function in ischemic rabbit myocardium. Circ. Res. **50**, 360–368.

Bush, L. R., Li, Y., Schlafer, M., Jolin, S. R. and Lucchesi, B. R. (1981): Protective effects of diltiazem during myocardial ischemia in isolated cat hearts. J. Pharmacol. Exp. Ther. **218**, 653–661.

Cheung, J. Y., Leaf, A. and Bonventre, J. V. (1984): Mechanism of protection by verapamil and nifedipine from anoxic injury in isolated cardiac myocytes. Am. J. Physiol. **246**, C323–C329.

Clark, R. E., Ferguson, T. B., West, P. N., Schuchleib, R. C. and Henry, P. D. (1977): Pharmacological preservation of the ischemic heart. Ann. Thorac. Surg. **24**, 307–314.

DeJong, J. W., Harmsen E., De Tombe, P. P. and Keijzer, E. (1982): Nifedipine reduces adenine nucleotide breakdown in ischemic rat heart. Eur. J. Pharmacol. **81**, 89–96.

Folch, J., Lees, M. and Sloane-Stanley, G. H. (1957): A simple method for the isolation and purification of total lipids from animal tissues. J. Biol. Chem. **226**, 497–509.

Hatefi, Y., Jurtshuk, P. and Haavik, A. G. (1961): Studies on the electron transport system. XXXII. Respiratory control in beef heart mitochondria. Arch. Biochem. Biophys. **94**, 148–153.

Henry, P. D., Schuchleib, R., Clark, R. E. and Perez, J. E. (1979): Effect of nifedipine on myocardial ischemia: analysis of collateral blood flow, pulsatile heat and regional muscle shortening. Am. J. Cardiol. **44**, 817–824.

Higgins, A. J. and Blackburn, K. J. (1984): Prevention of reperfusion damage in working rat hearts by calcium antagonists and calmodulin antagonists. J. Mol. Cell. Cardiol. **16**, 427–438.

Ichihara, K. and Abiko, Y. (1983): Effects of diltiazem and propranolol on irreversibility of ischemic cardiac function and metabolism in isolated, perfused rat heart. J. Cardiovasc. Pharmacol. **5**, 745–751.

Janis, R. A. and Scriabine, A. (1983): Sites of action of calcium channel inhibitors. Biochem. Pharmacol. **32**, 3499–3507.

Janis, R. A. and Triggle, D. J. (1983): New developments in calcium channel antagonists. J. Med. Chem. **26**, 775–785.

Jolly, S. R. and Gross, G. J. (1979): Effect of FR 7534, a new calcium antagonist on myocardial oxygen demand. Eur. J. Pharmacol. **54**, 289–293.

Katz, A. M. and Messineo, F. C. (1981): Lipid–membrane interactions and the pathogenesis of ischemic damage in the myocardium. Circ. Res. **48**, 1–7.

Lamprecht, W., Stein, P., Heinz, F. and Weisser, H. (1974): Creatine phosphate. In: Bergmeyer, H. U. (ed.) Methods of Enzymatic Analysis. Academic Press, New York. pp. 1777–1781.

Lands, W. E. M. (1960): Metabolism of glycerolipids. II. The enzymatic acylation of lysolecithin. J. Biol. Chem. **235**, 2233–2237.

Michalak, J., Kadziolka, A., Pruszkowska, R., Ledwozyw, A. and Madejczyk, A. (1988): Compensatory mechanism in erythrocyte lipids in patients with atherosclerosis. Lipids **23**, 476–480.

Miayazaki, Y., Nagai, S., Ogawa, K., Satake, T., Sugiyama, S. and Ozawa, T. (1984): The role of phospholipase in mitochondrial dysfunction after coronary reperfusion in the canine myocardium. Jpn. Circ. J. **48**, 498–505.

Nayler, W. G. (1982): Protection of the myocardium against postischemic reperfusion damage. J. Thorac. Cardiovasc. Surg. **84**, 897–905.

Nayler, W. G., Ferrari, R. and Williams, A. (1980): Protective effect of pretreatment with verapamil, nifedipine and propranolol on mitochondrial function in the ischemic and reperfused myocardium. Am. J. Cardiol. **46**, 242–249.

Neely, J. R. and Grotyohann, L. W. (1984): Role of glycolytic products in damage to ischemic myocardium. Dissociation of adenosine triphosphate levels and recovery of function of reperfused ischemic hearts. Circ. Res. **55**, 816–824.

Reibel, D. K. and Rovetto, M. J. (1978): Myocardial ATP synthesis and mechanical function following oxygen deficiency. Am. J. Physiol. **234**, H620–H624.

Ross-Nicholson, C., Currie, W. D. and Wechsler, A. S. (1978): Effects of verapamil on myocardial tolerance to ischemic arrest. Comparison to potassium arrest. Circulation **58**, 1119–1124.

Sokal, R. R. and Rohlf, F. J. (1981): Biometry. 2nd ed. W. H. Freeman, San Francisco.

Van Den Bosh, H. (1980): Intracellular phospholipase A. Biochim. Biophys. Acta **604**, 191–207.

Watts, J. A., Koch, C. D. and LaNoue, K. F. (1980): Effects of calcium antagonism on energy metabolism: calcium and heart function after ischemia. Am. J. Physiol. **238**, H909–H916.

Watts, J. A., Majorano, L. J. and Majorano, P. C. (1985): Protection by verapamil of globally ischemic rat hearts: energy preservation, a partial explanation. J. Mol. Cell. Cardiol. **17**, 797–804.

Weishaar, R. E., Ashikawa, K. and Bing, R. J. (1979): Effect of diltiazem, a calcium antagonist on myocardial ischemia. Am. J. Cardiol. **43**, 1137–1143.

Williams, L. T., Lefkovitz, R. J., Watanabe, A. M., Hathaway, D. R. and Besch, H. R. (1977): Thyroid hormone regulation of β-adrenergic receptor number. J. Biol. Chem. **252**, 2787–2791.

Williamson, J. R. and Corkey, B. E. (1969): Assays of intermediates of the citric acid cycle and related compounds by fluorometric enzyme methods. Meth. Enzymol. **13**, 434–512.

Williamson, J. R., Schaffer, S. W., Ford, C. and Safer, B. (1976): Contribution of tissue acidosis to ischemic injury in the perfused rat heart. Circulation **53**, 1–3.

Acta Veterinaria Hungarica 43 (2–3), pp. 321–333 (1995)

TOXICOLOGICAL STUDIES ON POTENTIATED IONOPHORES IN CHICKENS I. TOLERANCE STUDY

J. LEHEL, P. LACZAY, Zsuzsa MÓRA and G. SEMJÉN

Department of Pharmacology and Toxicology, University of Veterinary Science, H–1400 Budapest, P.O. Box 2, Hungary

(Received November 16, 1994)

The tolerance of chickens to monensin (12.5 mg/kg of feed) and maduramicin (3.0 mg/kg of feed) fed at a reduced dose in the presence of the antioxidant duokvin was studied in two experiments including 2 × 200 Tetra-82 broiler chickens. Tolerance was assessed by the appearance of clinical signs indicative of a toxic effect, the number of deaths, the groups' body weight gain, feed and drinking water intake, the aspartate aminotransferase (AST) and lactate dehydrogenase (LDH) activities, calcium ion, inorganic phosphate and total protein content of the blood plasma, the haematocrit value, and haemoglobin concentration. When applied at a dose that had proved to be optimum in the efficacy studies, neither the monensin-duokvin combination (12.5 mg monensin per kg of feed + 120 mg duokvin per kg of feed) nor the maduramicin-duokvin combination (3.0 mg maduramicin per kg of feed + 120 mg duokvin per kg of feed) exerted a statistically significant influence on the parameters tested.

Key words: Potentiated ionophore, monensin, maduramicin, tolerance, chicken

In broiler chickens raised on deep litter in large masses, regular supplementation of the feed with some anticoccidial agent is indispensable for the prevention of losses caused by poultry coccidiosis.

In the past two decades, the use of ionophore (polyether-type) antibiotics as chemoprophylactic agents has come to the fore all over the world. These compounds are effective against all *Eimeria* species pathogenic to fowl (Shumard and Callender, 1968; Reid et al., 1972; Mitrovic and Schildknecht, 1974; Danforth et al., 1977; Weppelman et al., 1977; Kantor and Schenkel, 1984; Varga, 1984, 1988; Salisch, 1987; Varga and Laczay, 1988; Folz et al., 1988). In contrast to synthetic compounds, ionophores induce the development of resistance only after a long period of use (Chapman, 1976; Hamet, 1989).

From the toxicological point of view, the use of ionophores is not without risk, as they may damage the cells of the host organism. The special literature contains numerous reports on cases of poisoning caused by ionophore antibiotics (Matsuoka, 1976; Howell et al., 1980; Dilov et al., 1981; Halvarson et al., 1982;

0236–6290/95/$ 4.00 © 1995 *Akadémiai Kiadó, Budapest*

Bourque et al., 1986; Galitzer et al., 1986; Morisse et al., 1986; Pietsch and Rüffle, 1986; Rollinson et al., 1987; Sályi et al., 1988; Papp et al., 1989), due to the overdosage of these compounds, their incomplete mixing into the diet (sedimentation, segregation), or their use on animal species other than those specified in the indications.

Ionophores are incompatible with numerous other chemotherapeutic agents (tiamulin, erythromycin, oleandomicin, certain sulfonamides) and dihydroquinoline derivatives of antioxidative effect. Of the latter, at the currently applied dose rate monensin, salinomycin and narasin are incompatible with the antioxidants XAX-M and duokvin, while maduramicin gives an interaction of low degree with duokvin (Prohászka et al., 1987; Laczay et al., 1989). At the same time, these interactions resulted in a conspicuous increase of anticoccidial efficacy, although the antioxidants themselves possessed no considerable anticoccidial activity (Varga, 1990). Infection experiments carried out in the laboratory with the strains *Eimeria tenella* and *E. mitis* revealed that the simultaneous administration of duokvin at a dose of 120 mg/kg of feed rendered it possible to reduce the dose of monensin from 100 mg to 10–12 mg/kg of feed and of maduramicin from 5 mg to 2.5–3.0 mg/kg of feed, i.e. that duokvin potentiated the effect of ionophorous anticoccidials (Varga et al., 1994).

The objective of these experiments was to study the tolerance of broiler chickens to the combined use of reduced doses of monensin or maduramicin with duokvin.

Materials and methods

Trial I: Monensin-duokvin combination

Experimental procedures

Two hundred Tetra-82 cockerel chicks were used in the experiment. The birds were weighed individually at day old, then assigned to one of five groups on the basis of their body weight (40 chicks per group). Four subgroups were formed within each group. The chicks were fattened in Delta type broiler batteries for 49 days. Feed and drinking water were available *ad libitum* throughout the experiment. During the rearing period, 24-hour illumination was ensured and the temperature of the rearing house was controlled continuously.

Monensin [MON] (Elancoban-100 medicated premix) was mixed into the feed (starter and grower diet) at a dose of 12.5 (group I), 20.0 (group II), 27.5 (group III) and 100 mg/kg of feed (group IV).

To the diet of groups I, II and III, duokvin [2,2-bis(2,2,4-trimethyl-1,2-dihydroquinoline-6-yl)-propane; Material Chemical Co-operative, Budapest, Hungary] was added at a dose of 120 mg/kg of feed, whereas that of group IV was

supplemented with EMQ at the dose of 120 mg/kg of feed. Group V served as untreated control. The finisher diet fed between day 43 and 49 contained neither monensin nor antioxidant.

Mortality was recorded throughout the experiment, and the dead chicks were necropsied and examined for any signs indicative of toxicity.

The chicks were weighed individually on days 21, 42 and 49. Feed and drinking water consumption was checked daily throughout the trial period.

On days 21 and 42, blood samples were taken from 10 cockerels per group, and assayed for haematocrit value, haemoglobin concentration, blood plasma aspartate aminotransferase (AST) and lactate dehydrogenase (LDH) activities as well as Ca^{2+}, inorganic phosphate and total protein content.

Haemoglobin concentration was determined by photometry using the cyanmethaemoglobin method, and the haematocrit value was measured with a Cellokrit-2 type haematocrit centrifuge.

Blood plasma AST and LDH activities were determined by optimized kinetic methods, using the appropriate Boehringer diagnostic tests, at 37 °C, with a Hitachi 705 type automatic chemical analyzer.

The Ca^{2+} content of the blood plasma was determined by the photometric measurement of the complex formed by Ca ions with o-cresol-phthalein in an alkaline medium, and total protein content by the biuret reaction. Both variables were determined with the help of a Hitachi 705 automatic chemical analyzer.

Inorganic phosphate concentration of the blood plasma was determined with the help of a Boehringer diagnostic test kit, by the photometric measurement of the colour intensity of the phosphate–ammonium molybdenate complex.

Statistical analysis of the results was done by Student's *t* test.

Trial II: Maduramicin-duokvin combination

Experimental procedures

The chickens were grouped, kept and fed as described for Trial I.

The starter and the grower diet fed to groups I–III were supplemented with 3.0, 4.5 and 6.0 mg maduramicin [MAD] (Cygro 1% medicated premix), respectively, and with 120 mg duokvin [DV] (Material Chemical Co-operative, Budapest, Hungary) per kg of feed. As positive control, group IV was fed a diet containing 5.0 mg maduramicin and 120 mg EMQ per kg of feed during the first 42 days of the trial. Group V served as untreated control.

The birds were weighed individually at 21, 42 and 49 days of age. On days 21 and 42, blood samples were taken from 10 chicks per group.

The feed consumption of each subgroup and the drinking water intake of each group of cockerels were determined daily throughout the trial period. Mor-

tality was recorded and the dead chicks were subjected to gross pathological examination.

Blood samples taken on days 21 and 42 were assayed for blood plasma AST and LDH activities, haematocrit value, and haemoglobin concentration. The calcium ion, inorganic phosphate and total protein concentrations of the blood plasma were also determined.

The haematologic and clinicochemical parameters were measured as described for Trial I.

Statistical analysis of the results was performed by Student's *t* test.

Results

Trial I: Monensin-duokvin combination

Clinical signs, mortality

None of the groups showed clinical signs indicative of toxicity during the experiment. The mortality rate was relatively low (5.0–7.5%) in all groups. No marked inter-group differences were demonstrable in mortality rate. Gross pathological examination of the dead birds did not reveal gross pathological changes (Table 1).

Body weight gain

Chicks of group I, fed the monensin-duokvin combination at a dose found to be optimum in the efficacy studies (12.5 mg monensin per kg of feed and 120 mg duokvin per kg of feed), showed a body weight gain practically identical with that of the control group (group V). Raising the dose of monensin to 20 mg/kg of feed (group II) exerted hardly any influence on body weight gain: by day 49 the body weight of group II chicks was only 0.4% less than that of the untreated control. On the other hand, if the dose of monensin was increased to 27.5 mg/kg of feed (group III), this resulted in a 5.5% lower body weight gain by the end of the experiment (this reduction in body weight gain was not statistically significant). The body weight gain of group IV chicks used as positive control and fed monensin at a dose rate of 100 mg/kg of feed was practically identical with that of the control group (Table 2).

Table 1

Mortality

Trial I: Monensin-duokvin combination

Feed		Number of animals	Mortality			
Drug	Dosage (mg/kg)		day 1–21	day 22–42	day 43–49	day 1–49
MON	12.5	40	1	1	0	2
DV	120					
MON	20.0	40	2	1	0	3
DV	120					
MON	27.5	40	1	2	0	3
DV	120					
MON	100	40	2	1	0	3
EMQ	120					
–	–	40	1	2	0	3

Trial II: Maduramicin-duokvin combination

Feed		Number of animals	Mortality			
Drug	Dosage (mg/kg)		day 1–21	day 22–42	day 43–49	day 1–49
MAD	3.0	40	0	2	0	2
DV	120					
MAD	4.5	40	2	1	0	3
DV	120					
MAD	6.0	40	1	2	0	3
DV	120					
MAD	5.0	40	0	1	0	1
EMQ	120					
–	–	40	1	0	1	2

Feed consumption, water intake

Compared with the control group, the feeding of 12.5 mg monensin and 120 mg duokvin per kg of the starter and grower diet (group I) exerted practically no influence on feed consumption and feed conversion efficiency.

The feed consumption of group II cockerels fed 20 mg/kg monensin and 120 mg/kg duokvin slightly decreased (by 1.4%) in the period when the birds were

fed the grower diet (days 22–42). At the same time, the consumption of finisher diet was 2.6% higher in group II than in the control group (compensation).

Group III chicks fed monensin at a dose of 27.5 mg/kg of feed consumed much less feed than the controls.

Chicks of group IV (100 mg monensin and 120 mg EMQ per kg of feed) consumed somewhat less feed than the control birds (Tables 3 and 4).

No significant differences were observed between the groups in drinking water intake ($P > 0.05$, Table 5).

Haematological and biochemical parameters

No statistically significant inter-group differences were observed in the haematological parameters (haematocrit value, haemoglobin concentration) and clinicochemical variables (blood plasma AST and LDH activities, calcium, inorganic P and total protein content) tested ($P > 0.05$).

Trial II: Maduramicin-duokvin combination

Clinical signs, mortality

None of the birds showed clinical signs indicative of toxicity during the experiment. The mortality data are shown in Table 1. No pathological changes suggestive of toxicity were found at necropsy. The mortality rate varied between 2.5 and 7.5% in the different groups. No appreciable differences occurred between the groups in mortality rate. Coccidiosis was not established as the cause of mortality in any of the cases.

Body weight gain

The average body weight gain of the different groups is presented in Table 2. The body weight gain of the group fed 3.0 mg/kg maduramicin and 120 mg/kg duokvin was identical with that of the control throughout the trial. At the same time, on day 21, 42 and 49 the body weight of chicks fed 4.5 mg maduramicin and 120 mg duokvin per kg of feed (group II) was by 1.8, 3.3 and 1.4% less, respectively, than that of the controls. These differences were not statistically significant. Compared to the control group, the body weight gain of cockerels fed 6.0 mg/kg maduramicin and 120 mg/kg duokvin (group III) was significantly lower: the difference was 6.1, 9.2 and 5.4% on day 21, 42 and 49, respectively. The body weight of birds fed a diet supplemented with 5.0 mg/kg maduramicin and 120 mg/kg EMQ (group IV) was hardly lower than that of the untreated controls. These differences (1.0, 0.4 and 0.7% on day 21, 42 and 49, respectively) were not statistically significant either.

Table 2

Body weight gain

Trial I: Monensin-duokvin combination

Feed		Body weight					
Drug	Dosage mg/kg	day 21		day 42		day 49	
		g	%	g	%	g	%
MON/DV	12.5/120	466.2 ± 50.3[a]	101.0	1468.2 ± 206.6[ab]	99.3	1779.0 ± 239.7[a]	100.4
MON/DV	20.0/120	462.2 ± 44.1[a]	100.1	1448.3 ± 185.0[ab]	98.0	1764.8 ± 235.5[a]	99.6
MON/DV	27.5/120	434.0 ± 43.3[b]	94.0	1390.8 ± 204.2[b]	94.1	1674.5 ± 239.5[a]	94.5
MON/EMQ	100/120	448.0 ± 42.9[ab]	97.0	1454.6 ± 150.9[ab]	98.4	1773.6 ± 187.3[a]	100.1
–	–	461.8 ± 56.6[a]	100.0	1477.9 ± 171.3[a]	100.0	1772.0 ± 194.8[a]	100.0

Trial II: Maduramicin-duokvin combination

Feed		Body weight					
Drug	Dosage mg/kg	day 21		day 42		day 49	
		g	%	g	%	g	%
MAD/DV	3.0/120	468.3 ± 64.3[ab]	99.8	1461.3 ± 177.4[a]	100.1	1790.4 ± 186.0[a]	100.2
MAD/DV	4.5/120	460.7 ± 60.5[ab]	98.2	1411.8 ± 192.9[ab]	96.7	1761.7 ± 196.8[ab]	98.6
MAD/DV	6.0/120	440.6 ± 61.4[b]	93.9	1324.6 ± 204.8[b]	90.8	1689.8 ± 206.1[b]	94.6
MAD/EMQ	5.0/120	464.8 ± 52.0[ab]	99.0	1454.2 ± 169.0[a]	99.6	1777.4 ± 174.1[a]	99.3
–	–	469.3 ± 53.9[a]	100.0	1459.5 ± 175.1[a]	100.0	1786.6 ± 183.2[a]	100.0

[a, b] Values within a column not followed by a common superscript are significantly different ($P < 0.05$)

Table 3

Feed consumption

Trial I: Monensin-duokvin combination

Feed		Feed consumption					
Drug	Dosage mg/kg	day 1–21		day 22–42		day 43–49	
		g	%	g	%	g	%
MON/DV	12.5/120	840 ± 17[a]	100.6	2178 ± 24[a]	99.4	906 ± 16[a]	101.0
MON/DV	20.0/120	840 ± 15[a]	100.6	2162 ± 35[ab]	98.6	920 ± 24[a]	102.6
MON/DV	27.5/120	821 ± 23[a]	98.3	2108 ± 38[b]	96.2	884 ± 39[a]	98.6
MON/EMQ	100/120	826 ± 17[a]	98.9	2166 ± 19[a]	98.8	914 ± 17[a]	101.9
–	–	835 ± 19[a]	100.0	2192 ± 20[a]	100.0	897 ± 21[a]	100.0

Trial II: Maduramicin-duokvin combination

Feed		Feed consumption					
Drug	Dosage mg/kg	day 1–21		day 22–42		day 43–49	
		g	%	g	%	g	%
MAD/DV	3.0/120	845 ± 13[a]	99.4	2152 ± 19[a]	99.4	926 ± 21[a]	100.7
MAD/DV	4.5/120	837 ± 22[a]	98.5	2106 ± 24[b]	97.3	938 ± 28[a]	102.0
MAD/DV	6.0/120	816 ± 28[a]	96.0	2008 ± 38[c]	92.7	938 ± 24[a]	102.0
MAD/EMQ	5.0/120	842 ± 16[a]	99.1	2150 ± 17[a]	99.3	925 ± 16[a]	100.5
–	–	850 ± 18[a]	100.0	2165 ± 24[a]	100.0	920 ± 18[a]	100.0

[a, b, c] Values within a column not followed by a common superscript are significantly different ($P < 0.05$)

Table 4

Feed conversion

Trial I: Monensin-duokvin combination

Feed		Feed conversion					
Drug	Dosage mg/kg	day 1–21		day 1–42		day 1–49	
		kg/kg	%	kg/kg	%	kg/kg	%
MON/DV	12.5/120	1.80 ± 0.04^{a}	99.4	2.06 ± 0.03^{a}	100.5	2.21 ± 0.03^{a}	100.0
MON/DV	20.0/120	1.82 ± 0.03^{ab}	100.6	2.07 ± 0.04^{a}	101.0	2.22 ± 0.04^{ab}	100.5
MON/DV	27.5/120	1.89 ± 0.05^{b}	104.4	2.11 ± 0.05^{a}	103.0	2.28 ± 0.04^{b}	103.2
MON/EMQ	100/120	1.84 ± 0.04^{ab}	101.7	2.06 ± 0.04^{a}	100.5	2.20 ± 0.03^{a}	99.5
–	–	1.81 ± 0.03^{a}	100.0	2.05 ± 0.03^{a}	100.0	2.21 ± 0.03^{a}	100.0

Trial II: Maduramicin-duokvin combination

Feed		Feed conversion					
Drug	Dosage mg/kg	day 1–21		day 1–42		day 1–49	
		kg/kg	%	kg/kg	%	kg/kg	%
MAD/DV	3.0/120	1.80 ± 0.03^{a}	99.4	2.05 ± 0.04^{a}	99.0	2.19 ± 0.03^{a}	99.5
MAD/DV	4.5/120	1.82 ± 0.04^{a}	100.6	2.08 ± 0.03^{ab}	100.5	2.20 ± 0.04^{a}	100.0
MAD/DV	6.0/120	1.85 ± 0.04^{a}	102.2	2.13 ± 0.04^{b}	102.9	2.23 ± 0.05^{a}	101.4
MAD/EMQ	5.0/120	1.18 ± 0.04^{a}	100.1	2.06 ± 0.03^{a}	99.5	2.20 ± 0.03^{a}	100.0
–	–	1.18 ± 0.04^{a}	100.0	2.07 ± 0.04^{a}	100.0	2.20 ± 0.04^{a}	100.0

[a, b,] Values within a column not followed by a common superscript are significantly different ($P < 0.05$)

Table 5

Water intake

Trial I: Monensin-duokvin combination

Feed		Water intake			
Drug	Dosage mg/kg	day 16–20		day 36–40	
		ml	%	ml	%
MON	12.5	112 ± 8[a]	98.2	220 ± 5[ab]	97.8
DV	120				
MON	20.0	108 ± 6[ab]	94.7	212 ± 8[ab]	94.2
DV	120				
MON	27.5	98 ± 10[b]	86.0	198 ± 10[c]	88.0
DV	120				
MON	100	108 ± 8[ab]	94.7	217 ± 8[ab]	96.4
EMQ	120				
–	–	114 ± 6[a]	100.0	225 ± 7[a]	100.0

Trial II: Maduramicin-duokvin combination

Feed		Water intake			
Drug	Dosage mg/kg	day 16–20		day 36–40	
		ml	%	ml	%
MAD	3.0	116 ± 8[a]	96.7	226 ± 8[a]	101.8
DV	120				
MAD	4.5	110 ± 7[ab]	91.7	215 ± 10[a]	96.8
DV	120				
MAD	6.0	102 ± 9[b]	85.0	194 ± 12[b]	87.4
DV	120				
MAD	5.0	120 ± 8[a]	100.0	220 ± 8[a]	99.1
EMQ	120				
–	–	120 ± 9[a]	100.0	222 ± 9[a]	100.0

[a, b, c] Values within a column not followed by a common superscript are significantly different ($P < 0.05$)

Feed consumption, water intake

The feed consumption and water intake data are summarized in Tables 3 and 4. The feed consumption of cockerels fed a diet supplemented with 3.0 mg/kg maduramicin + 120 mg/kg duokvin (group I) was the same as that of the control birds (group IV). The feed consumption of group II (fed 4.5 mg/kg maduramicin + 120 mg/kg duokvin) and group III (fed 6.0 mg/kg maduramicin + 120 mg/kg

duokvin) showed a statistically not significant decrease (1.5 and 4%, respectively) in the starting period (day 1–21) and a statistically significant decrease (3.2 and 7.3%, respectively) in the growing period. Compared to the untreated control, the feed consumption of the birds treated earlier was 0.5–2.0% higher in the finishing period.

The feed conversion efficiency of the different groups followed the trend described for feed consumption.

The average water intake data of the chicks are presented in Table 5. The water intake of group I (3.0 mg/kg maduramicin + 120 mg/kg duokvin) did not differ markedly from that of the control group. Between day 16–20 and day 36–40 of the trial, the drinking water intake of birds fed 4.5 mg/kg maduramicin + 120 mg/kg duokvin (group II) was 8.3% and 3.2% lower, respectively, than that of the control group. These differences were not statistically significant. At the same time, a statistically significant decrease of water intake occurred in group III fed a diet supplemented with 6.0 mg/kg maduramicin + 120 mg/kg duokvin: water intake of that group was by 15.0% (day 16–20) and 12.6% (day 36–40) lower than in the control.

Haematological and biochemical parameters

No statistically significant difference was demonstrable between the treated groups and the untreated control group in the haematological and biochemical parameters tested ($P > 0.05$).

Discussion

Ionophore antibiotics are known to be preparations of a narrow therapeutic spectrum and liable to overdosage. In a 30–50% overdose they usually reduce the body weight gain of chicks and, when administered in a 2- to 3-fold dose, they may produce fatal toxicosis (Damron et al., 1977; Howell et al., 1980; Keshavarz and McDougald, 1982; Christmas and Harms, 1983; Pietsch and Rüffle, 1986; Harms and Buresh, 1987).

In the first trial it was found that the monensin-duokvin combination, when applied at the therapeutic dose found to be optimum in efficacy studies (12.5 mg/kg monensin + 120 mg/kg duokvin), causes no clinical signs, does not increase the mortality rate, does not decrease the birds' body weight gain, feed consumption and water intake, and exerts practically no influence on the haematological and clinicochemical parameters tested. Raising the dose of monensin to 20 mg/kg of feed hardly affected the body weight gain: at 49 days the body weight of the group fed that diet was only 0.4% less than that of the untreated control. At the same time, the body weight gain decreased by 5.5% in the group fed a monensin dose elevated to 27.5 mg/kg of feed.

In the second trial, none of the test parameters showed a significant change in the group fed 3.0 mg/kg maduramicin + 120 mg/kg duokvin. At the same time, a 50% elevation of the dose of maduramicin (4.5 mg/kg maduramicin + 120 mg/kg duokvin) resulted in a slight (1.4–8.3%) decrease in the average body weight, feed consumption and water intake data with respect to the untreated control group. These differences were not statistically significant. The differences found for the other parameters were even smaller. A 100% elevation of the maduramicin dose (6.0 mg/kg maduramicin + 120 mg/kg duokvin) caused significant differences (5.4–15.0%; $P < 0.05$) in average body weight gain and water intake as compared to the control group. Differences obtained for the other parameters were not statistically significant. The mortality rate did not change in any of the cases.

The results of these trials indicate that the monensin-duokvin combination can be safely used at the recommended dose (12.5 mg/kg monensin + 120 mg/kg duokvin). That combination is somewhat better tolerated by chickens than the currently used monensin dose (100 mg/kg of feed). When fed at the recommended 3.0 mg maduramicin + 120 mg/kg duokvin dose, the maduramicin-duokvin combination does not impair the birds' general health status and production parameters.

References

Bourque, J. G., Smart, M. and Wobeser, G. (1986): Monensin toxicity in lambs. Can. Vet. J. **27,** 379–399.

Chapman, H. D. (1976): *Eimeria tenella* in chickens: studies on resistance to the anticoccidial drugs monensin and lasalocid. Vet. Parasitol. **2,** 187–196.

Christmas, R. B. and Harms, R. H. (1983): The effect of protein level and/or other selected dietary nutrients on broiler chicks receiving monensin. Nutr. Rep. Int. **28,** 1105–1109.

Damron, B. L., Harms, R. H., Arafa, A. S. and Janky, D. M. (1977): The effect of dietary lasalocid or monensin in the presence of roxarsone and graded methionine levels on broiler performance and processing characteristics. Poultry Sci. **56,** 1487–1491.

Danforth, H. D., Ruff, M. D., Reid, W. M. and Miller, R. (1977): Anticoccidial activity of salinomycin in battery raised broiler chickens. Poultry Sci. **56,** 926–932.

Dilov, P., Dimitrov, S. and Dzhurov, A. (1981): Toxicity of monensin sodium for piglets. Vet. Med. Nauki **18,** 55–63.

Folz, S. D., Lee, B. L. Nowakowski, L. H. and Conder, G. A. (1988): Anticoccidial evaluation of halofuginone, lasalocid, maduramicin, monensin and salinomycin. Vet. Parasitol. **28,** 1–9.

Galitzer, S., Oehme, F. W., Bartley, E. E. and Dayton, A. D. (1986): Lasalocid toxicity in cattle: acute clinicopathological changes. J. Anim. Sci. **62,** 1308–1316.

Halvarson, D. A., Van Dijk, C. and Brown, P. (1982): Ionophore toxicity in turkey breeders. Avian Diseases **26,** 634–639.

Hamet, N. (1989): Activity of some anticoccidial drugs to turkey coccidiosis. 5th Int. Coccidiosis Conference Proc., Tours, 265–266.

Harms, R. H. and Buresh, R. E. (1987): Influence of salinomycin on the performance of broiler chicks. Poultry Sci. **66,** 51–54.

Howell, J., Hanson, J., Onderka, D. and Harries, W. N. (1980): Monensin toxicity in chickens. Avian Diseases **24,** 1050–1053.

Kantor, S. and Schenkel, R. H. (1984): CL 259,971: a potent new polyether anticoccidial. 1. Battery efficacy and safety. Poultry Sci. **63,** 1497–1505.

Keshavarz, K. and McDougald, L. (1982): Anticoccidial drugs: growth and performance depressing effects in young chickens. Poultry Sci. **61,** 699–705.

Laczay, P., Simon, F., Barócsai, Gy. and Vörös, G. (1989): Study of the compatibility of XAX-M with certain ionophorous antibiotics in chickens (in Hungarian, with English abstract). Magyar Állatorvosok Lapja **44,** 359–363.

Matsuoka, T. (1976): Evaluation of monensin toxicity in the horse. J. Am. Vet. Med. Ass. **169,** 1098–1100.

McDougald, L. R., Wang, G. T., Kantor, S., Shenkel, R. and Quarles, C. (1987): Efficacy of maduramicin against ionophore-tolerant field isolates of coccidia in broilers. Avian Diseases **31,** 302–308.

Mitrovic, M. and Schildknecht, E. G. (1974): Anticoccidial activity of lasalocid (X-537A) in chicks. Poultry Sci. **53,** 1448–1455.

Mitrovic, M., Schildknecht, E. G. and Marusich, W. L. (1975): Comparative anticoccidial activity and compatibility of lasalocid in broiler chickens. Poultry Sci. **54,** 757–762.

Morisse, J. P., Boilletot, E. and Maurice, R. (1986): Toxicite du narasin chez le lapin: Étude de quelques cas cliniques. Ann. Med. Vet. **130,** 101–107.

Papp, L., Karsai, F., Sályi, G., Bagó, Gy., Kántás, K. and Siver, L. (1989): Narasin poisoning in dogs (in Hungarian with English abstract). Magyar Állatorvosok Lapja **44,** 179–185.

Pietsch, W. and Rüffle, E. (1986): Zur Toxizität des Monensin und zu Problemen seines Einsatzes im Broilerfutter. Mh. Vet.-Med. **41,** 851–854.

Prohászka, L., Hajdú, É., Dworschák, E. and Rozsnyai, T. (1987): Growth depression in broiler chicks caused by incompatibility of feed ingredients. Acta Vet. Hung. **35,** 349–358.

Reid, W. M., Kowalski, L. and Rice, J. (1972): Anticoccidial activity of monensin in floor-pen experiments. Poultry Sci. **51,** 139–146.

Rollinson, J., Taylor, F. G. R. and Chesney, J. (1987): Salinomycin poisoning in horses. Vet. Rec. **124,** 126–128.

Salish, H. (1987): Wirksamkeit des Antikokzidiums Maduramicin-ammonium unter Versuchs- und Feldbedingungen. Arch. Gelflügelk. **51,** 190–196.

Sályi, G., Szabó, E., Bagó, Gy., Bánhidi, Gy. and Szilágyi, M. (1988): Narasin poisoning in turkeys. Acta Vet. Hung. **36,** 107–114.

Shumard, R. F. and Callender, M. E. (1968): Monensin, a new biologically active compound. VI. Anticoccidial activity. Antimicrob. Agents Chemother. **7,** 367–377.

Varga, I. (1984): Battery studies on the anticoccidial efficacy of narasin in chickens. (in Hungarian with English abstract) Magyar Állatorvosok Lapja **39,** 459–462.

Varga, I. (1988): Battery studies on the anticoccidial efficacy of maduramicin ammonium in chickens. (in Hungarian with English abstract) Magyar Állatorvosok Lapja **43,** 285–289.

Varga, I. (1990): Potentiation of ionophorous anticoccidial with antioxidant Duokvin. Abstracts, VIIth International Congress of Parasitology, Paris, p. 866.

Varga, I. and Laczay, P. (1988): Untersuchungen über die Wirksamkeit von Maduramicin (Cygro) unter Labor- und Betriebsbedingungen bei Broilern. Tagung der Fachgruppe der DVG in Verbindung mit der WVPA. Wien, 18–21 Juli, 1988.

Varga, I., Laczay, P., Lehel, J., Móra, Zs., Romváry, A. and Fekete, J. (1994): Potentiation of ionophorous anticoccidials with dihydroquinolines: Battery trial against *Eimeria tenella* in chickens. Int. J. Parasitol. **24,** 689–694.

Weppelman, R. M., Olson, G., Smith, D. A., Tamas, T. and Van Iderstine, A. (1977): Comparison of anticoccidial efficacy, resistance and tolerance of narasin, monensin and lasalocid in chicken battery trials. Poultry Sci. **56,** 1550–1559.

Acta Veterinaria Hungarica 43 (2–3), pp. 335–345 (1995)

TOXICOLOGICAL STUDIES ON POTENTIATED IONOPHORES IN CHICKENS II. COMPATIBILITY STUDY

J. LEHEL, P. LACZAY, Zsuzsa MÓRA and G. SEMJÉN

Department of Pharmacology and Toxicology, University of Veterinary Science, H–1400 Budapest, P.O. Box 2, Hungary

(Received November 16, 1994)

Two trials were carried out on a total of 2 × 360 Tetra-82 broiler chickens to study how the presence of the antioxidant duokvin as potentiating agent influenced the compatibility of reduced doses of monensin (12.5 mg/kg of feed) or maduramicin (3.0 mg/kg of feed) with other chemotherapeutic agents (tiamulin, erythromycin, sulfaquinoxaline, sulfachlorpyrazine, flumequine, tylosin, kitasamycin) widely used in broiler rearing. Compatibility was assessed on the basis of the appearance of clinical signs suggestive of toxic interaction, the mortality rate, body mass gain, feed consumption and drinking water intake, and changes in AST and LDH activities of the blood plasma. The monensin-duokvin combination (12.5 mg monensin/kg of feed + 120 mg duokvin/kg of feed) was found to be compatible with erythromycin, sulfaquinoxaline, sulfachlorpyrazine, flumequine, tylosin and kitasamycin. For tiamulin, a slight incompatibility was observed; however, this was much less severe than that found for monensin administered at a dose of 100 mg/kg of feed. The maduramicin-duokvin combination (3.0 mg maduramicin/kg of feed + 120 mg duokvin/kg of feed) was compatible with all the compounds tested; thus, it can be safely applied also in combination with tiamulin.

Key words: Potentiated ionophore, monensin, maduramicin, compatibility, chicken

The use of ionophore antibiotics involves some risks due to incompatibility with certain compounds. Monensin, salinomycin and narasin are incompatible with tiamulin, erythromycin, oleandomycin and certain sulfonamides (Goff et al., 1980; Hilbrich and Trautwein, 1980; Horrox, 1980; Hamet and Bennejean, 1980; Weisman et al., 1980; Fink, 1981; Frigg et al., 1983; Mazlum et al., 1985; Laczay et al., 1987, 1989*a*, 1989*b*). Of the above compounds, lasalocid shows incompatibility only with sulfadimethoxine (Frigg et al., 1983). Maduramicin is less incompatible with tiamulin and duokvin (Prohászka et al., 1987; Laczay et al., 1989*c*, 1990*a*) and it is compatible with erythromycin and sulfonamides.

In addition to the compounds listed above, ionophores may enter into interaction with certain antioxidants as well. At a dose usually applied for prevention,

0236–6290/95/$ 4.00 © 1995 *Akadémiai Kiadó, Budapest*

monensin, salinomycin and narasin are incompatible with XAX-M and with the antioxidant duokvin (Prohászka et al., 1987; Laczay et al., 1989*c*).

These interactions resulted in a conspicuous increase of anticoccidial efficacy, although the antioxidants themselves possessed no anticoccidial activity (Varga, 1990). In laboratory experiments with *Eimeria tenella* and *E. mitis* strains it was found that the simultaneous administration of duokvin at a dose of 120 mg/kg of feed enabled a reduction of the monensin dose from 100 mg to 10–12 mg/kg of feed.

In the trials reported here, monensin and maduramicin administered at reduced doses in combination with the antioxidant duokvin were studied for compatibility with other chemotherapeutic agents in broiler chickens.

Materials and methods

Trial I: Monensin-duokvin combination

Experimental procedures

In this experiment, the compatibility of the monensin-duokvin combination with erythromycin (ERY), flumequine (FLU), kitasamycin (KIT), sulfachlorpyrazine (SCP), sulfaquinoxaline (SQ), tiamulin (TIA) and tylosin (TYL) was studied.

A total of 360 four-week-old Tetra-82 sexed cockerel chicks, kept in broiler rearing batteries of Delta type, were used. Nine groups (each containing 40 chickens) were formed, and each group was subdivided into four subgroups.

From day-old age, the cockerels were fed broiler starter and then grower diets containing 12.5 mg monensin [MON] (Elancoban-100 medicated premix, BCR Works) and 120 mg duokvin antioxidant [DV] (Material Chemical Co-operative) per kg of feed. Cockerel chicks of group IX (control) received a diet free from the monensin-duokvin combination. Feed and drinking water (from a self-waterer system) were available *ad libitum*.

At 28 days of age (on day 1 of the trial) the chicks were weighed and marked individually and blood samples were taken from 10 birds in each group. On day 5, the chicks were again weighed individually and blood samples were taken from 10 birds in each group as previously.

Between day 5 and 8 of the trial, chicks of groups I–VII received the following chemotherapeutic agents, dissolved in the drinking water: tiamulin 250 mg/l (Dynamutilin 45% granulate, HAGE), erythromycin 200 mg/l (erythromycin thiocyanate 50%, Phylaxia), sulfaquinoxaline 400 mg/l (100%, Phylaxia), sulfachlorpyrazine 450 mg/l (100%, Phylaxia), flumequine 125 mg/l (Imequil 10% pulvis, Rhône Merieux), tylosin 500 mg/l (Tylan soluble pulvis, Chemical Works of G. Richter), kitasamycin 500 mg/l (Trubin pulvis, Phylaxia).

The chicks were again weighed individually after the treatments (on day 9) and at the end of the subsequent 4-day period of observation (on days 13 and 17), and blood samples were taken from 10 birds in each group. Feed consumption (by subgroup) and drinking water intake (by group) were determined on each day of the trial (on days 1–16), the number of deaths was recorded daily, and the dead chicks were necropsied.

Blood samples taken on days 1, 5, 9, 13 and 17 of the trial were assayed for blood plasma AST and LDH activities by optimised kinetic procedures, using the appropriate Boehringer diagnostic tests, at 25 °C in a Hitachi 705 type automatic chemical analyser.

Trial II: Maduramicin-duokvin combination

Experimental procedures

A total of 360 Tetra-82 sexed cockerels were used. The birds were grouped, fed and kept as described for Trial I.

From day-old age, chicks of groups I–VIII were fed broiler starter and then grower diets containing 3.0 mg maduramicin [MAD] (Cygro 1% medicated premix, Cyanamid) and 120 mg duokvin per kg of feed. Cockerel chicks of group IX (untreated control) received a diet free from the maduramicin-duokvin combination but otherwise of identical content. Feed and drinking water were available *ad libitum*.

At 28 days of age (on day 1 of the trial) the chicks were weighed and marked individually and blood samples were taken from 10 birds in each group. On days 1–4 the chicks were monitored for clinical signs. On day 5, they were again weighed individually and blood samples were taken from 10 birds in each group as previously.

Between day 5 and 8 of the trial, chicks of groups I–VII received the following chemotherapeutic agents, dissolved in the drinking water: tiamulin 250 mg/l, erythromycin 200 mg/l, sulfaquinoxaline 400 mg/l, sulfachlorpyrazine 450 mg/l, flumequine 125 mg/l, tylosin 500 mg/l, kitasamycin 500 mg/l. The treatments were followed by two four-day periods of observation between days 9–12 and 13–16, respectively. The chicks were weighed individually on days 9, 13 and 17 of the trial and blood samples were taken from 10 birds in each group. Feed consumption (by subgroup) and drinking water intake (by group) were determined on each day of the trial by measuring the quantity of leftovers. The number of deaths was recorded daily and the dead chicks were necropsied.

Blood samples taken on days 1, 5, 9, 13 and 17 of the trial were assayed for blood plasma AST and LDH activities as described for Trial I.

Results

Trial I: Monensin-duokvin combination

Clinical signs, mortality

None of the birds showed signs of toxicity, indicative of drug interaction, during the trial. Only one death occurred: in group VI, treated with a combination of monensin-duokvin + tylosin, one chick died on the first day of the treatment period (Table 1). Prior to death, the chick did not show clinical signs indicative of a toxic interaction. No pathological changes suggestive of toxicosis were found at necropsy.

Table 1

Mortality

Trial I: Monensin-duokvin combination

Treatment		No. of animals	Mortality
Feed	Water		day 1–16
MON/DV	TIA	40	0
MON/DV	ERY	40	0
MON/DV	SQ	40	0
MON/DV	SCP	40	0
MON/DV	FLU	40	0
MON/DV	TYL	40	1
MON/DV	KIT	40	0
MON/DV	–	40	0
–	–	40	0

Trial II: Maduramicin-duokvin combination

Treatment		No. of animals	Mortality
Feed	Water		day 1–16
MAD/DV	TIA	40	0
MAD/DV	ERY	40	0
MAD/DV	SQ	40	0
MAD/DV	SCP	40	0
MAD/DV	FLU	40	0
MAD/DV	TYL	40	0
MAD/DV	KIT	40	1
MAD/DV	–	40	0
–	–	40	0

Body weight gain

Between day 1 and 4, no significant difference occurred between the groups.

Between day 5 and 8 of the trial, the body weight gain of group I (monensin-duokvin + tiamulin) was significantly ($p < 0.001$) lower (by 24.5%) than that of the control group.

In the observation period between day 9 and 12 that difference was no longer demonstrable, and chicks fed monensin-duokvin + tiamulin showed a 6.3% higher body mass gain, which compensated for the previous difference.

Table 2

Body weight gain

Trial I: Monensin-duokvin combination

Treatment		Body weight gain (g/animal/day)			
Feed	Water	day 1–4	day 5–8	day 9–12	day 13–16
MON/DV	TIA	48 ± 6	40 ± 20***	64 ± 12	62 ± 10
MON/DV	ERY	50 ± 7	52 ± 12	58 ± 9	61 ± 8
MON/DV	SQ	48 ± 5	53 ± 8	59 ± 10	61 ± 10
MON/DV	SCP	47 ± 6	51 ± 8	58 ± 11	61 ± 10
MON/DV	FLU	49 ± 5	51 ± 7	60 ± 12	62 ± 9
MON/DV	TYL	51 ± 7	54 ± 8	59 ± 10	63 ± 8
MON/DV	KIT	50 ± 6	52 ± 7	58 ± 9	61 ± 9
MON/DV	–	48 ± 6	51 ± 7	58 ± 9	63 ± 8
–	–	49 ± 6	53 ± 8	60 ± 10	63 ± 9

*** Significantly different from the untreated control ($P < 0.001$)

Trial II: Maduramicin-duokvin combination

Treatment		Body weight gain (g/animal/day)			
Feed	Water	day 1–4	day 5–8	day 9–12	day 13–16
MAD/DV	TIA	46 ± 8	47 ± 9	58 ± 12	60 ± 8
MAD/DV	ERY	45 ± 8	49 ± 9	57 ± 9	61 ± 7
MAD/DV	SQ	47 ± 6	51 ± 8	57 ± 8	61 ± 7
MAD/DV	SCP	48 ± 6	50 ± 6	56 ± 7	60 ± 8
MAD/DV	FLU	48 ± 7	51 ± 9	56 ± 8	63 ± 9
MAD/DV	TYL	46 ± 5	52 ± 10	58 ± 8	63 ± 8
MAD/DV	KIT	49 ± 6	51 ± 6	56 ± 7	61 ± 9
MAD/DV	–	49 ± 6	53 ± 8	57 ± 7	62 ± 8
–	–	47 ± 7	51 ± 8	56 ± 7	62 ± 8

Not significantly different from the untreated control in either period ($P > 0.05$)

In the subsequent period of observation (days 13–16) no important difference was demonstrable between the group treated with the monensin-duokvin combination plus tiamulin and the untreated control group in body weight gain.

For the other groups (II–IX), no statistically significant difference was found in body weight gain during the trial (Table 2).

Feed consumption, water intake

No considerable difference was found between the groups in feed consumption and drinking water intake on the first four days.

During treatment (days 5–8), the feed consumption and drinking water intake of a single group (group I, receiving monensin-duokvin + tiamulin) differed from that of the control group in a statistically significant degree: the feed consumption and drinking water intake of group I was 17.2% ($p < 0.001$) and 11.3% ($p < 0.05$) lower, respectively, than those of the control group (group IX).

Such difference was no longer demonstrable during the two 4-day periods of observation, and the values of both parameters were nearly identical in the two groups.

For the other groups (II–IX), no major differences in feed consumption and drinking water intake were observed either in the treatment or in the observation periods (Table 3).

Biochemical parameters

In cockerel chicks of group I, receiving the monensin-duokvin + tiamulin combination, blood plasma AST activity was slightly increased after the treatment period (on day 9). That increase was statistically significant ($p < 0.05$). Increased AST activity was no longer demonstrable on day 13 and day 17. The values measured on days 1, 5, 9, 13 and 17 of the trial in chicks of the other groups (groups II–IX) showed no major difference in blood plasma AST activity (Table 4).

As a result of the simultaneous administration of the monensin-duokvin combination and tiamulin, blood plasma LDH activity was slightly elevated on days 9, 13 and 17 of the trial. However, that change was statistically not significant ($p > 0.05$). In the other groups (II–IX), no significant change occurred in blood plasma LDH activity during the trial (Table 4).

Table 3

Feed consumption and water intake

Trial I: Monensin-duokvin combination

Treatment		Feed consumption (g/animal/day)				Water intake (ml/animal/day)			
Feed	Water	day 1–4	day 5–8	day 9–12	day 13–16	day 1–4	day 5–8	day 9–12	day 13–16
MON-DV	TIA	100 ± 14	101 ± 13**	129 ± 14	141 ± 11	188 ± 11	197 ± 17*	237 ± 20	258 ± 18
MON-DV	ERY	99 ± 12	115 ± 9	124 ± 16	141 ± 10	196 ± 15	224 ± 10	244 ± 16	266 ± 9
MON-DV	SQ	97 ± 14	118 ± 7	126 ± 13	143 ± 9	193 ± 10	217 ± 8	235 ± 15	257 ± 10
MON-DV	SCP	101 ± 16	116 ± 13	124 ± 13	139 ± 13	194 ± 11	210 ± 10	230 ± 10	260 ± 12
MON-DV	FLU	102 ± 9	118 ± 15	126 ± 15	144 ± 10	204 ± 13	211 ± 11	228 ± 12	256 ± 13
MON-DV	TYL	103 ± 11	122 ± 10	131 ± 12	144 ± 8	192 ± 11	216 ± 8	233 ± 15	264 ± 10
MON-DV	KIT	104 ± 12	119 ± 13	127 ± 10	140 ± 13	196 ± 8	220 ± 10	238 ± 11	258 ± 9
MON-DV	–	101 ± 13	118 ± 12	126 ± 12	140 ± 10	188 ± 10	212 ± 10	232 ± 10	264 ± 8
–	–	103 ± 13	122 ± 12	130 ± 11	144 ± 8	194 ± 11	222 ± 8	238 ± 10	261 ± 9

*, ** Significantly different from the untreated control (* $P < 0.05$, ** $P < 0.01$)

Trial II: Maduramicin-duokvin combination

Treatment		Feed consumption (g/animal/day)				Water intake (ml/animal/day)			
Feed	Water	day 1–4	day 5–8	day 9–12	day 13–16	day 1–4	day 5–8	day 9–12	day 13–16
MAD-DV	TIA	96 ± 11	108 ± 13	129 ± 12	144 ± 10	196 ± 10	209 ± 17	244 ± 18	260 ± 12
MAD-DV	ERY	94 ± 13	111 ± 12	125 ± 10	141 ± 11	190 ± 13	221 ± 15	240 ± 12	255 ± 10
MAD-DV	SQ	99 ± 11	115 ± 12	127 ± 11	143 ± 10	199 ± 11	218 ± 13	237 ± 13	255 ± 13
MAD-DV	SCP	97 ± 10	113 ± 13	124 ± 10	139 ± 13	195 ± 11	222 ± 13	242 ± 10	258 ± 10
MAD-DV	FLU	95 ± 13	113 ± 12	126 ± 12	145 ± 12	191 ± 13	220 ± 13	246 ± 11	255 ± 13
MAD-DV	TYL	95 ± 11	115 ± 10	130 ± 8	147 ± 12	201 ± 15	226 ± 13	256 ± 12	267 ± 10
MAD-DV	KIT	100 ± 12	115 ± 10	126 ± 11	141 ± 9	204 ± 13	221 ± 12	240 ± 13	258 ± 12
MAD-DV	–	101 ± 13	117 ± 11	127 ± 10	142 ± 8	200 ± 12	230 ± 12	246 ± 10	263 ± 10
–	–	98 ± 12	115 ± 13	126 ± 11	146 ± 9	195 ± 13	225 ± 13	241 ± 11	257 ± 10

Not significantly different from the untreated control in either period ($P > 0.05$)

Table 4

AST and LDH activities

Trial I: Monensin-duokvin combination

Treatment		AST activity (U/l)					LDH activity (U/l)				
Feed	Water	day 1	day 5	day 9	day 13	day 17	day 1	day 5	day 9	day 13	day 17
MON-DV	TIA	68 ± 5	66 ± 7	77 ± 12*	74 ± 13	69 ± 9	434 ± 82	427 ± 65	477 ± 87	486 ± 81	456 ± 67
MON-DV	ERY	61 ± 5	64 ± 6	62 ± 6	66 ± 7	66 ± 7	411 ± 63	419 ± 51	426 ± 48	431 ± 50	418 ± 37
MON-DV	SQ	67 ± 8	68 ± 6	68 ± 8	70 ± 8	68 ± 5	430 ± 51	419 ± 60	408 ± 61	422 ± 45	418 ± 51
MON-DV	SCP	64 ± 6	64 ± 8	63 ± 5	67 ± 10	67 ± 6	439 ± 57	421 ± 52	433 ± 48	422 ± 45	419 ± 52
MON-DV	FLU	65 ± 6	64 ± 7	61 ± 6	66 ± 7	68 ± 7	415 ± 49	430 ± 59	430 ± 52	427 ± 45	425 ± 46
MON-DV	TYL	63 ± 7	66 ± 6	66 ± 6	64 ± 5	67 ± 7	440 ± 60	426 ± 48	438 ± 49	446 ± 61	429 ± 45
MON-DV	KIT	62 ± 5	64 ± 7	65 ± 8	66 ± 7	63 ± 5	418 ± 63	426 ± 50	430 ± 55	415 ± 48	426 ± 56
MON-DV	–	67 ± 7	65 ± 5	64 ± 7	64 ± 6	65 ± 6	433 ± 47	441 ± 59	435 ± 55	431 ± 60	431 ± 51
–	–	64 ± 4	65 ± 7	62 ± 6	66 ± 9	66 ± 7	419 ± 52	426 ± 39	431 ± 58	422 ± 48	427 ± 51

* Significantly different from the base data (average of day 1 and day 5) ($P < 0.05$)

Trial II: Maduramicin-duokvin combination

Treatment		AST activity (U/l)					LDH activity (U/l)				
Feed	Water	day 1	day 5	day 9	day 13	day 17	day 1	day 5	day 9	day 13	day 17
MAD-DV	TIA	53 ± 6	55 ± 7	58 ± 9	56 ± 6	59 ± 10	405 ± 73	413 ± 61	395 ± 49	393 ± 53	415 ± 72
MAD-DV	ERY	54 ± 3	56 ± 4	56 ± 5	53 ± 6	57 ± 8	429 ± 79	420 ± 61	413 ± 57	397 ± 68	420 ± 80
MAD-DV	SQ	57 ± 6	60 ± 6	60 ± 7	55 ± 10	58 ± 8	421 ± 71	417 ± 58	402 ± 65	401 ± 64	404 ± 48
MAD-DV	SCP	58 ± 8	58 ± 5	56 ± 6	59 ± 8	62 ± 7	416 ± 67	421 ± 72	410 ± 60	403 ± 58	422 ± 68
MAD-DV	FLU	54 ± 6	55 ± 8	55 ± 6	51 ± 7	57 ± 8	435 ± 74	425 ± 66	428 ± 68	423 ± 61	431 ± 67
MAD-DV	TYL	55 ± 5	57 ± 6	58 ± 7	55 ± 6	57 ± 6	418 ± 59	409 ± 69	422 ± 61	411 ± 58	429 ± 70
MAD-DV	KIT	57 ± 6	56 ± 6	59 ± 7	60 ± 9	60 ± 8	409 ± 66	420 ± 58	417 ± 55	427 ± 69	433 ± 71
MAD-DV	–	60 ± 7	60 ± 6	58 ± 6	61 ± 7	61 ± 7	422 ± 63	421 ± 55	415 ± 60	409 ± 63	426 ± 70
–	–	55 ± 7	58 ± 8	58 ± 6	58 ± 8	60 ± 7	416 ± 56	405 ± 61	425 ± 58	412 ± 61	427 ± 58

Not significantly different from the base data (average of day 1 and day 5) in either group ($P > 0.05$)

Trial II: Maduramicin-duokvin combination

Clinical signs, mortality

None of the birds showed signs suggestive of a toxic drug interaction during the trial. The mortality data are shown in Table 1. Only a single bird died during the 16-day trial, in the group treated with a combination of maduramicin-duokvin + kitasamycin. Prior to death, the chick did not show clinical signs indicative of a toxic interaction. No pathological changes suggestive of toxicosis were found at necropsy.

Body weight gain

Data on the body weight gain of the different groups are presented in Table 2. Between day 1 and 4, no significant difference occurred between the groups in body weight gain ($p > 0.05$). During treatment (days 5–8) the body weight gain of group I chicks (treated with maduramicin-duokvin + tiamulin) was slightly lower than that of the untreated controls. That difference (7.9%), however, was not significant. During the two four-day periods of observation "a compensation" of that difference could be seen. In the other groups (II–IX) only slight changes were observed, and even these were compensated for later.

Feed consumption, water intake

Data on feed consumption and drinking water intake are shown in Table 3. On the first four days, no considerable difference was found between the groups in that respect. During treatment (days 5–8), feed consumption and drinking water intake were slightly lower in group I receiving the maduramicin-duokvin + tiamulin combination than in the control group; however, that nonsignificant difference was compensated for during the period of observation (days 9–12 and days 13–16). For the other groups (II–IX), no major differences in feed consumption and drinking water intake were observed either in the treatment or in the observation periods as compared to the untreated control chicks.

Biochemical parameters

The changes found in blood plasma AST and LDH activities are presented in Table 4. Statistically significant differences were not found in any of the groups for either of the two enzymes ($p > 0.05$).

Discussion

Ionophore (polyether-type) antibiotics are the compounds most widely used for the prevention of coccidiosis in broiler flocks all over the world. At the same time, the simultaneous use of other chemotherapeutic agents is indispensable for the control of numerous bacterial infections. The combined use of ionophores and other chemotherapeutic drugs has revealed that monensin, salinomycin and narasin is incompatible with erythromycin, oleandomycin, certain sulfonamides, tiamulin, and with certain antioxidants (Goff et al., 1980; Hilbrich and Trautwein, 1980; Horrox, 1980; Hamet and Bennejean, 1980; Weisman et al., 1980; Fink, 1981; Frigg et al., 1983; Mazlum et al., 1985; Laczay et al., 1987, 1989*a*, 1989*b*, 1989*d*).

When administered at its current dose of 100 mg/kg of feed, monensin is expressly incompatible with tiamulin. Simultaneous use of the two compounds results in severe depression, anorexia and muscular weakness, and deaths may also occur (Hilbrich and Trautwein, 1980; Frigg et al., 1983; Laczay et al., 1987).

When used simultaneously with tiamulin, the monensin-duokvin combination (12.5 mg monensin + 120 mg duokvin/kg of feed) caused a transient reduction in body weight gain and feed consumption and a slight elevation in blood plasma AST activity; however, these changes were much less expressed than in the case when a full dose of monensin was administered simultaneously with tiamulin (Laczay et al., 1990*b*). In this trial, no mortalities occurred, in contrast to when a high dose of monensin (100 mg/kg of feed) was given together with tiamulin. At its currently applied dose rate, monensin is incompatible with erythromycin and sulfaquinoxaline (Frigg et al., 1983; Laczay et al., 1987). At the same time, the monensin-duokvin combination used in the present trial proved to be fully compatible with these compounds. Monensin applied at a reduced dose was compatible with tylosin, kitasamycin and flumequine as well.

At its currently applied dose rate (5.0 mg/kg of feed), maduramicin shows a slight incompatibility with tiamulin (Laczay et al., 1989c, 1990a). After lowering the dose and adding the antioxidant duokvin, that incompatibility ceased to exist. When applied at a dose rate of 3.0 mg/kg of feed, maduramicin slightly decreased the chickens' body weight gain, feed consumption and drinking water intake; however, that difference was statistically not significant and was subsequently compensated for in a short time. When applied at a dose of 5.0 mg/kg of feed, maduramicin is compatible with erythromycin, flumequine, kitasamycin, sulfachlorpyrazine, sulfaquinoxaline and tylosin (Laczay et al., 1989*c*). It remains compatible with all these compounds also when administered at a lowered dose (3.0 mg/kg of feed) in combination with the antioxidant duokvin (120 mg/kg of feed).

The results of this study indicate that the monensin-duokvin combination is compatible with erythromycin, sulfaquinoxalin, sulfachlorpyrazine, flumequine,

tylosin and kitasamycin. It is slightly incompatible with tiamulin; however, this incompatibility is much less expressed than in the case when monensin is applied at its full dose. The maduramicin-duokvin combination is compatible with all the compounds tested and, thus, can be safely administered in combination with these agents.

References

Fink, J. (1981): Arzneimittelinteraktionen: Ein Beitrag zur Frage der Veträglichkeit von Monensin und Tiamulin. Dtsch. Tierärtztl. Wschr. **88,** 228–231.

Frigg, M., Broz, J. and Weber, G. (1983): Compatibility studies of ionophore anticoccidials with various antibiotics and chemotherapeutics in broiler chicks. Arch. Gelflügelk. **47,** 215–220.

Goff, S., James, T. M. and Merino, M. (1980): Tiamulin in the chicken: A summary review of compatibility studies. 29th West. Poultry Disease Conf. and 14th Calif. Health Symp. Proc. 119–124.

Hamet, N. and Bennejean, G. (1980): Innocuité des associations tiamuline-monensin, tiamulin-hlofuginone vis-á-vis de pulets. 6. Europ. Gelflügelkonferenz Proc., Vol. II., Hamburg, pp. 356–362.

Hilbrich, P. and Trautwein, G. (1980): Tiamulin-Monensin-Intoxikation Beim Huhn. Prakt. Tierarzt, **61,** 2622–2625.

Horrox, N. E. (1980): Monensin-tiamulin interaction risk to poultry. Vet. Rec. **106,** 278.

Laczay, P., Bozzay, L., Simon, F., Lehel, J., Dobos-Kovács, M., Móra, Zs. and Ribiczei, P. (1987): Study of the compatibility between monensin and other chemotherapeutics in broilers (in Hungarian, with English abstract). Magyar Állatorvosok Lapja **42,** 109–114.

Laczay, P., Simon, F., Móra, Zs. and Lehel, J. (1989*a*): Study of the compatibility of salinomycin and narasin with other chemotherapeutics in broilers (in Hungarian, with English abstract). Magyar Állatorvosok Lapja **44,** 173–177.

Laczay, P., Simon, F., Barócsai, Gy. and Vörös, G. (1989*b*): Study of the compatibility of XAX-M with certain ionophorous antibiotics in chickens (in Hungarian, with English abstract). Magyar Állatorvosok Lapja **44,** 359–363.

Laczay, P., Simon, F. and Lehel, J. (1989*c*): Study of the maduramycin tolerance in broilers (in Hungarian, with English abstract). Magyar Állatorvosok Lapja **44,** 529–532.

Laczay, P., Simon, F., Móra, Zs. and Lehel, J. (1989*d*): Zur Kompatibilität der neueren Ionophor-Antikokzidika mit anderen Chemotherapeutika bei Broilern. Dtsch. Tierärztl. Wschr. **96,** 433–472.

Laczay, P., Simon, F., Móra, Zs. and Lehel, J. (1990*a*): Komparative Untersuchungen über die Verträglichkeit der ionophoren Antikokzidia mit Tiamulin bei Broilern. Arch. Geflügelk. **54,** 129–132.

Laczay, P., Simon, F., Móra, Zs. and Lehel, J. (1990*b*): Study on biochemical characteristics of the toxic interaction between monensin and other chemotherapeutics, as well as antioxidants in broilers (in Hungarian, with English abstract). Magyar Állatorvosok Lapja, **45,** 103–107.

Mazlum, Z., Pickles, R. W., Pradella, G. and Pagnani, R. (1985): Interaction between monensin, narasin or salinomycin and the antibiotics erytromycin, chloramphenicol or tylozin in broiler chicks. Clin. Vet. **108,** 95–104.

Prohászka, L., Hajdú, É., Dworschák, E. and Rozsnyai, T. (1987): Growth depression in broiler chicks caused by incompatibility of feed ingredients. Acta Vet. Hung. **35,** 349–358.

Varga, I. (1990): Potentiation of ionophorous anticoccidials with antioxidant Duokvin. Abstracts, VIIth International Congress of Parasitology, Paris, p. 866.

Weisman, Y., Schlosberg, A. and Egyed, M. N. (1980): Acute poisoning in turkeys caused by incompatibility of monensin and tiamulin. Vet. Res. Commun. **4,** 231–235.

Acta Veterinaria Hungarica 43 (2–3), pp. 347–354 (1995)

TOXICOLOGICAL STUDIES ON POTENTIATED IONOPHORES IN CHICKENS III. ELECTROTOXICOLOGICAL INVESTIGATIONS

J. LEHEL and P. LACZAY

Department of Pharmacology and Toxicology, University of Veterinary Science, H–1400 Budapest, P.O. Box 2, Hungary

(Received November 16, 1994)

The effect exerted by the simultaneous administration of tiamulin (40 mg/kg body mass) and a lowered dose (12 mg/kg of feed) of monensin combined with the antioxidant duokvin on the conductivity of peripheral nerve and the electrophysiological function of the heart was studied in two trials comprising 3 × 6 broiler chickens. For the study of peripheral nerve function, chickens were anaesthetized with pentobarbital sodium, the sciatic nerve (n. ischiadicus) was exposed in the sciatic arc, and bipolar stimulating and efferent electrodes were inserted directly beside the nerve, at an average distance of 20 mm from each other. For the recordings, the nerve was stimulated with 16 supramaximal impulses of 0.02 ms duration, applied at 15-s intervals. The responses given to the stimulus were recorded with the help of a polygraph and evaluated with a computer in online mode. For the study of cardiac function, the chickens were anaesthetized with methomidate. The electrocardiograms were taken in Einthoven's lead II with the help of needle electrodes, and then evaluated with computer in online mode. In control chickens, maximum conduction velocity was 30.70 ± 0.52 m/s on the average. That value did not change after a single treatment with monensin-duokvin + tiamulin, while it underwent a slight but statistically significant decrease after two treatments. Studies on the electrical function of the heart revealed no signs of dysfunction in either of the treated groups as compared to the control. The result indicate that no toxic interaction manifesting itself in electrotoxicological changes occurred in chickens between tiamulin and monensin administered at a reduced dose in combination with the antioxidant duokvin.

Key words: Potentiated ionophore, monensin, tiamulin, ECG, peripheral nerve, chicken

Previous studies have revealed that the simultaneous administration of monensin and tiamulin markedly decreased the conduction velocity of the peripheral nerve and simultaneously increased the duration of the relative and absolute refractory period. Simultaneous administration of monensin and tiamulin causes slight changes also in the electric function of the heart by disturbing ventricular

0236–6290/95/$ 4.00 © 1995 *Akadémiai Kiadó, Budapest*

depolarization and repolarization and, as a consequence, prolonging intraventricular impulse conduction (Laczay et al., 1990*a*).

The objective of these trials was to study whether the simultaneous use of tiamulin and a reduced dose (12 mg/kg of feed) monensin combined with the antioxidant duokvin influenced the electrophysiological function of the peripheral nervous system and the heart.

Materials and methods

Trial I: Investigation of peripheral nerve function

Experimental procedures

Eighteen 4-week-old Tetra-82 broiler chicks were used in the trial. The birds were kept in broiler rearing batteries of Delta type and had been fed a diet free from anticoccidials and other medicaments since day-old age.

At 28 days of age, the chicks were weighed individually and divided into three groups (6 chicks/group). Chicks of group I received a single treatment while those of group II two treatments, given on two consecutive days, with 1 mg/bwkg monensin [MON] (Elancoban-100 medicated premix, BCR Works) and 9 mg/bwkg duokvin [DV] (antioxidant, Material Chemical Co-operative) administered individually, in the form of pills, and with tiamulin [TIA] (Dynamutilin 45% water-soluble granulate, HAGE) administered through a tube at a dose of 40 mg/bwkg. The chicks of group III served as untreated control.

The electrophysiological studies were performed 24 h after the last monensin-duokvin + tiamulin treatment (group I: day 2; group II: day 3; group III, control: day 1) on chicks anaesthetized by the intravenous administration of pentobarbital sodium (Nembutal). After separating the m. biceps femoris from the m. semitendinosus, the n. ischiadicus was exposed in the arcus ischiadicus. Bipolar stimulating and efferent electrodes were inserted directly beside the nerve, at an average distance of 20 mm from each other.

To measure conduction velocity, the nerve was stimulated with 16 supramaximal electric impulses of 0.02 ms duration, administered at 15-s intervals with the help of an ST-12 stimulator (Medicor, Budapest). By heating with an infrared lamp, the temperature of the nerve was kept at 39.5 ± 0.1 °C. Temperature was checked with a contact thermometer before the recordings. The nerve was kept moist with physiological saline.

The responses given to stimulation were recorded with the help of an R612 Dynograph Recorder (Sensormedics) and evaluated in online mode with an IBM compatible computer connected with the polygraph. The computerized processing of conduction velocity was performed according to the method of Anderson et al. (1981) with the help of Digivoice computer programmes. The action potentials

were analyzed and the values of maximum and peak conduction velocity were determined (Fig. 1).

The statistical analysis of the results was performed by Student's t-test.

Trial I: Investigation of heart function

Experimental procedures

The chicks were grouped, kept, fed and treated as described for Trial I.

Electrocardiography was carried out 24 h after the last treatment (group I: day 2; group II: day 3; group III, control: day 1), on chicks anaesthetized with methomidate (Hypnodil) administered intravenously at a dose of 30 mg/bwkg.

The electric potentials were recorded in Einthoven's lead II with the help of needle electrodes connected with an 8-channel polygraph (612 Dynograph Recorder, Sensormedics) according to the method described by Sturkie (1949) and Szabuniewicz (1967). The recorded electrocardiograms (Fig. 2) were evaluated with an online Digivoice computer programme. The following components of the ECG were analyzed: the amplitude of the P, R, S and T waves, the duration of the PR and ST segments and of the RS complex, the TP interval, and the heart rate. As, in a manner typical of the chicken ECG (Sturkie, 1949), in the majority of the chicks the T and the P wave could not be reliably distinguished, the duration of these waves were not analyzed separately.

Statistical evaluation of the results was performed by Student's t-test.

Results

Trial I: Investigation of peripheral nerve function

The conduction velocity values of the peripheral nerve are summarized in Table 1. In the control chicks, the maximum conduction velocity of the n. ischiadicus was 30.70 ± 0.52 m/s. This value did not change after a single treatment with monensin-duokvin + tiamulin (30.48 ± 0.47 m/s) and slightly decreased (28.37 ± 1.35 m/s) after two treatments; however, due to the low standard deviation even that small decrease proved to be statistically significant.

The values of peak conduction velocity did not deviate significantly from the control in either of the treated groups (group I: 16.08 ± 1.19; group II: 15.64 ± 0.74; group III, control: 16.24 ± 1.12).

The average duration of the action potentials did not change considerably.

Trial II: Investigation of heart function

The parameters characterizing the electrophysiological function of the heart are summarized in Table 2.

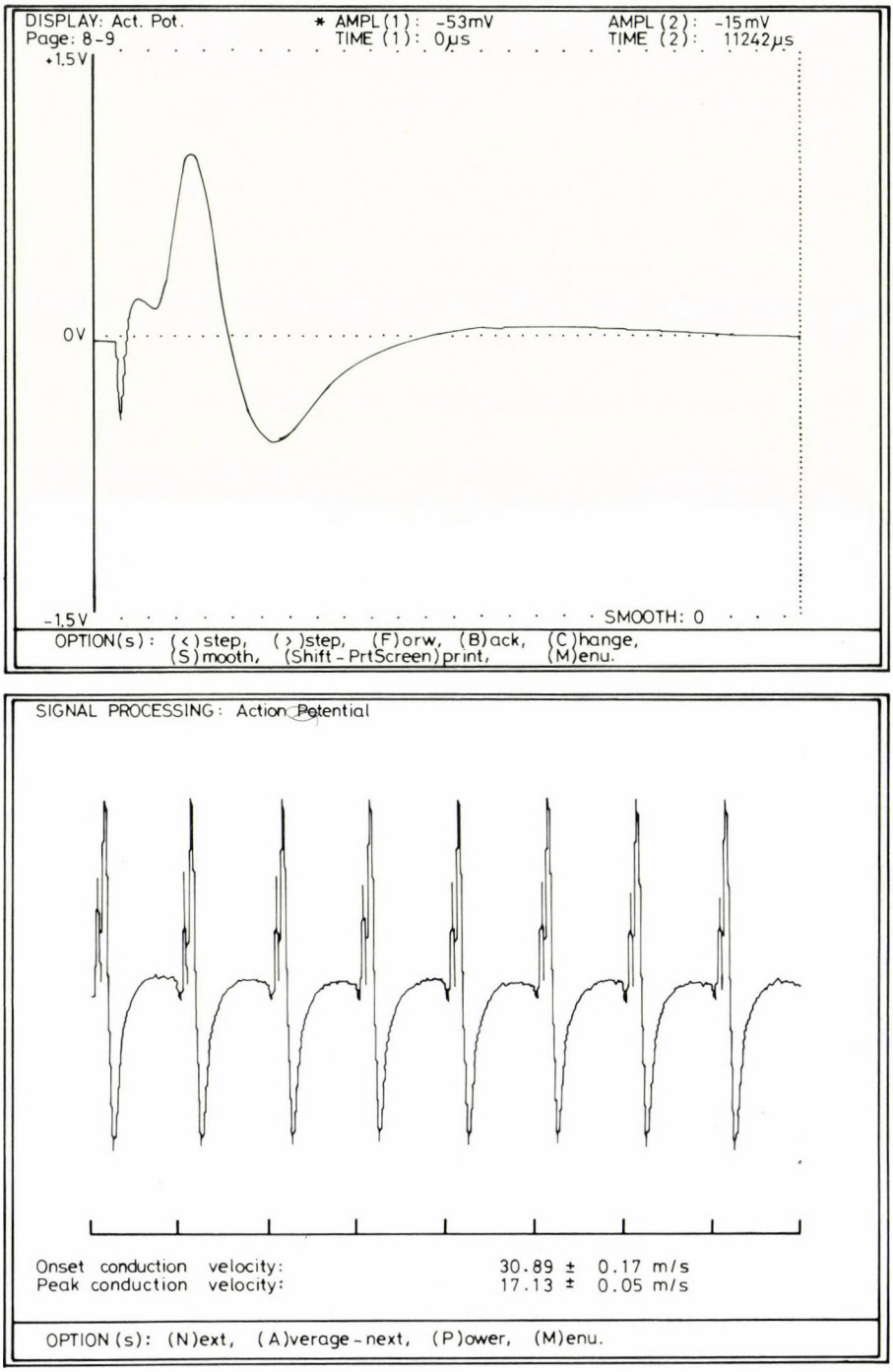


Fig. 1. Recording and evaluation of the action potential

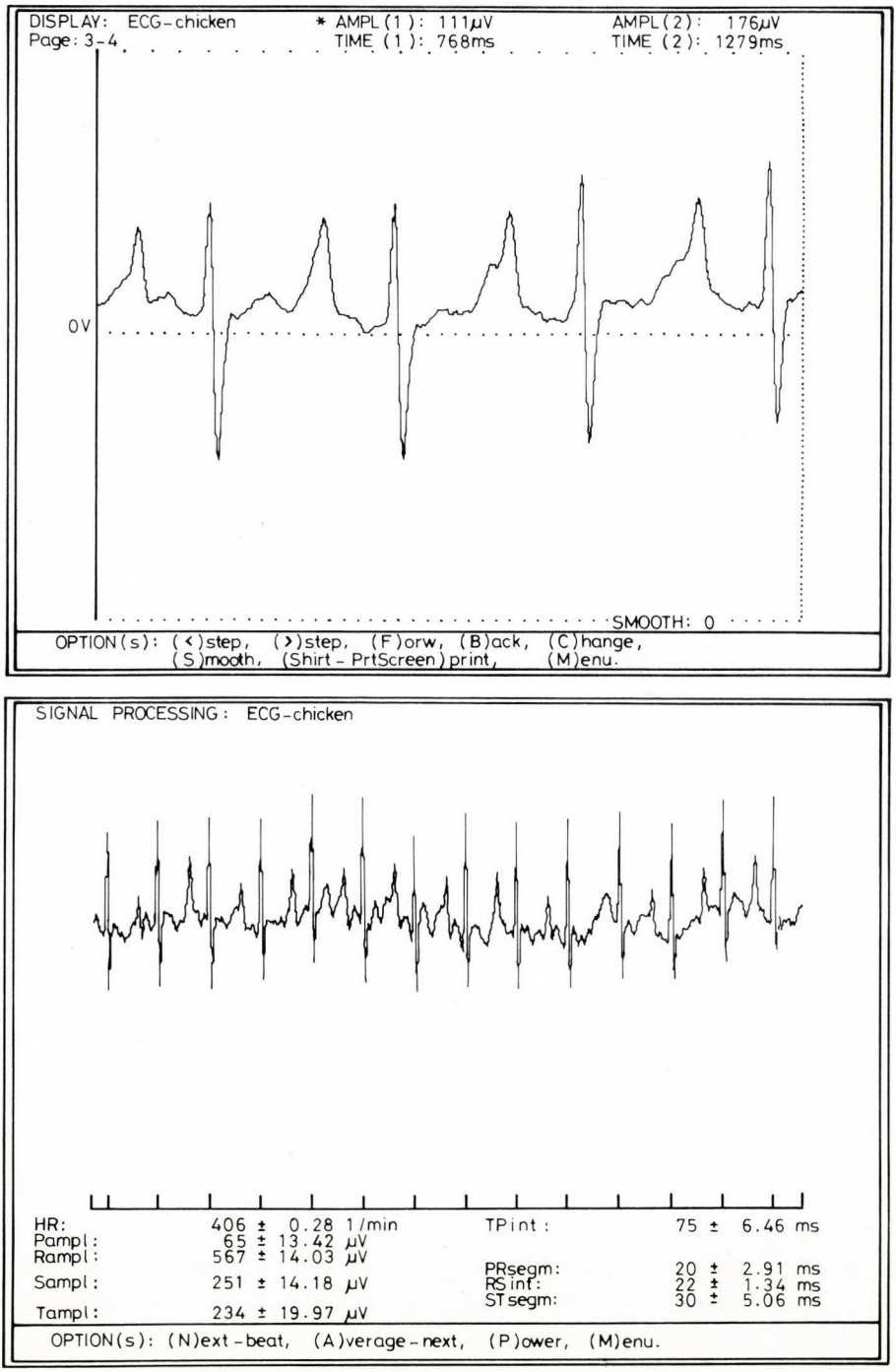


Fig. 2. Recording and analysis of the electrocardiogram

Table 1

Influence on the conduction velocity

Treatment	Onset conduction velocity (m/s)	Peak conduction velocity (m/s)
MON-DV + TIA 1×	30.48 ± 0.47	16.08 ± 1.19
MON-DV + TIA 2×	28.37 ± 1.35**	15.64 ± 0.74
Control	30.70 ± 0.52	16.24 ± 1.12

** Significantly different from the control group ($P < 0.01$)
MON: monensin; DV: Duokvin; TIA: tiamulin

No significant changes were found in the studied waves, segments and complexes of the ECG after either a single or a twofold administration of monensin, duokvin and tiamulin as compared to the control.

Discussion

The objective of these trials was to determine the effects exerted by the simultaneous administration of tiamulin with a reduced dose (12 mg/kg of feed) monensin plus duokvin on the conduction velocity of a peripheral nerve and on the bioelectric function of the heart.

According to the results, a single treatment with monensin-duokvin + tiamulin did not change the conduction velocity of the peripheral nerve, while two treatments caused a small decrease.

In earlier studies, a single treatment and two treatments with monensin (100 mg/kg of feed) and tiamulin (40 mg/kg of feed) caused a 25% and 28% decrease, respectively, in peripheral nerve conduction velocity as compared to values measured in control chickens (Laczay et al., 1990*a*).

The toxic interaction between ionophore anticoccidials and tiamulin is probably due to the accumulation of toxic metabolites arising from the biotransformation of ionophore compounds. These metabolites are more toxic than the parent molecule (Laczay et al., 1990*b*). Ionophore antibiotics are ion-carrier compounds which markedly enhance the transfer of different monovalent and bivalent cations across biological membranes.

Monensin belongs to the category of so-called monovalent ionophores which can form complexes primarily with Na^+ ions and stimulate their transfer through membranes. It increases the intracellular concentration of Na^+ and decreases that of K^+. These changes are accompanied by a simultaneous rise in the concentration of bound Ca^{2+} in the mitochondria and sarcoplasmic reticulum.

Table 2

Changes in the electrocardiogram

Treatment	P amplitude (mV)	R amplitude (mV)	S amplitude (mV)	T amplitude (mV)	Frequency (min)	PR segment (msec)	RS interval (msec)	ST segment (msec)	TP interval (msec)
MON-DV + TIA 1×	77.0 ± 16.0	491.3 ± 82.0	441.1 ± 73.6	391.0 ± 76.2	443.4 ± 23.6	17.4 ± 3.1	24.1 ± 2.0	23.1 ± 3.5	72.8 ± 6.7
MON-DV + TIA 2×	54.6 ± 14.8	465.3 ± 138.8	409.0 ± 75.4	303.0 ± 73.1	414.0 ± 35.7	16.0 ± 3.2	24.6 ± 2.2	24.7 ± 3.5	71.0 ± 24.2
Control	75.2 ± 17.5	450.5 ± 102.4	420.1 ± 80.2	350.2 ± 75.3	430.2 ± 40.1	18.3 ± 3.5	23.5 ± 2.4	23.3 ± 3.2	68.0 ± 6.4

MON: monensin; DV: Duokvin; TIA: tiamulin

The induction and spread of the action potential, as well as depolarization and repolarization basically depend on changes of the membrane potential and the extracellular as well as intracellular Na^+ and K^+ concentration determining the former. Major changes of the latter markedly affect the induction and spread of the action potential as well as depolarization and repolarization.

Ionophore compounds also cause changes in the release of acetylcholine acting as a neurotransmitter at synapses. In a nerve-muscle preparation subjected to the effect of ionophores (monensin, lasalocid) the release of acetylcholine initially increases and then, due to the depletion of stores, it decreases (Charlton et al., 1980; Meiri et al., 1981; Person and Kuhn, 1979).

Clinical signs (muscular weakness, motor disturbances) may appear already at the initial stage of the toxic interaction between monensin and tiamulin (on days 2–3). However, at that time the morphological changes are not so severe yet that they alone could account for the clinical signs observed. In the initial phase of the interaction, the dysfunction of the peripheral nerve probably plays an important role in their development.

In the trial reported here, clinical signs (motor disturbances, muscular weakness) indicative of a toxic interaction were not observed, which is consistent with the peripheral nerve conduction velocity values measured. The slight decrease found in peripheral nerve conduction velocity of chicks treated twice did not cause clinically perceptible changes.

The results obtained for cardiac function indicate that the simultaneous administration of tiamulin with a reduced dose of monensin combined with duokvin does not cause considerable changes in the bioelectric function of the heart.

References

Anderson, R. J., Parker, J. M. and Loeb, A. L. (1981): A microprocessor system for controlling stimulation and on-line analysis of electrophysiological parameters. J. Electrophys. Tech., **7,** 9–21.

Charlton, M. P., Thompson, C. S., Atwood, H. L. (1980): Synaptic transmission and intracellular sodium: Ionophore-induced sodium loading of nerve terminals. Neurosci. Lett., **16,** 193–196.

Laczay, P., Simon, F. and Lehel, J. (1990*a*): Effect of the simultaneous application of monensin and tiamulin on the function of peripheral nerves in broiler chickens (in Hungarian, with English abstract). Magyar Állatorvosok Lapja **45,** 287–290.

Laczay, P., Simon, F. and Lehel, J. (1990*b*): Untersuchungen über den Einfluss von Monensin, Tiamulin bzw. der gleichzeitigen Applikation der beiden Substanzen auf die mikrosomalen mischfunktionellen Oxygenasen und auf die Peroxidbildung bei Broilern. Dtsch. Tierärztl. Wschr. **97,** 354–357.

Meiri, H., Erulkar, S. D. and Lerman, T. (1981): The action of the sodium ionophore, monensin on transmitter release at the frog neuromuscular junction. Brain. Res. **204,** 204–208.

Person, R. J. and Kuhn, J. A. (1979): Depression of spontaneous and ionophore induced transmitter release by ruthenium red at the neuromuscular junction. Brain. Res. Bull. **4,** 669–674.

Sturkie, P. D. (1949): The electrocardiogram of the chicken. Am. J. Vet. Res. **10,** 168–175.

Szabunievicz, M. (1967): The electrocardiogram of the chicken. Southwest. Vet. **20,** 287–294.

Acta Veterinaria Hungarica 43 (2–3), pp. 355–358 (1995)

EFFECTS OF REPEATED ORAL DOSES OF DIKAMIN D (2,4-D-AMINE Na) ON RATS

L. VÁRNAGY[1], I. SOMLYAY[2], P. BUDAI[1] and T. VARGA[1]

[1]Department of Agrochemical Hygiene, Institute of Plant Protection; [2]Department of Chemistry, Georgikon Faculty, Pannon University of Agricultural Sciences, H–8361 Keszthely, P.O. Box 71, Hungary

(Received September 12, 1994)

The toxic effects of repeated, increasing oral doses of Dikamin D (72% 2,4-D-amine Na), a broad leave herbicide product used world-wide, were evaluated on rats by the method of Lim et al. (1961). A comparison of the determined acute oral LD_{50} and the calculated subchronic oral LD_{50} values revealed a definite tolerance of the experimental animals to the test compound. This finding indicates that repeated oral treatment is capable of increasing the test animal's metabolizing capacity, which accounts for the development of tolerance.

Key words: Herbicide, repeated doses, rat, 2,4-D-amine

In the assessment and evaluation of the toxicological characteristics of any pesticide, the determination of oral subchronic toxicity using repeated doses provides information on possible health hazards that are likely to arise from repeated exposure over a limited period of time. When considering mammalian toxicity data, the results obtained from oral administration are the most important with respect to those chemicals which have the potential e.g. to bioaccumulate. Although the literature contains some data on the toxicological characteristics of 2,4-D-amine (Rowe and Hymas, 1954; Erne, 1966; Khanna and Fang, 1966; Way, 1969; Heath et al., 1972; Hill and Camardese, 1986), our aim was to obtain own data on the acute and subchronic toxicity of the test material, as that would make it possible to calculate the correct dose of the agent for the purpose of long-term studies.

Materials and methods

Experimental conditions. The certified test material Dikamin D (72% 2,4-D-amine Na), a liquid herbicide, was supplied by Nitrokémia Industries (Fűzfőgyártelep, Hungary). As vehicle, distilled water was applied. SPF Wistar laboratory rats, kept in a GLP certified conventional test facility, were used for the study. The animal room was programmed for a 12-hour light cycle per day. The

0236–6290/95/$ 4.00 © 1995 *Akadémiai Kiadó, Budapest*

experimental animals were allocated to polypropylene cages, five animals per cage. They were fed Altromin rat food, and tap-water was available to them as drinking water.

Determination of the acute oral LD_{50}. Six groups of female rats with a starting body weight range of 231–277 g and three groups of male rats weighing 204–229 g (n = 6 per group) were used in the study (Table 1). The animals were treated orally by intragastric intubation after 16 h of food withdrawal. After treatment, the clinical signs, the body weight gains and the mortality rates were recorded over a 14-day observation period.

Table 1

Acute oral LD_{50} of Dikamin D in male and female rats

Dose µl/kg	Sex	No. of treated animals	No. of deaths	Mean body weight gain (g) during the observation
0	F	6	0	33 + 4.3
820	F	6	0	31 + 3.7
940	F	6	1	55 + 6.2
1070	F	6	2	47 + 5.1
1230	F	6	3	51 + 5.8
1400	F	6	6	–
LD_{50} = 1145.5 µl/kg (1040 – 1285 µl/kg), Y = –41.3 + 15.13 log(X)				
0	M	6	0	23 + 3.2
940	M	6	3	22 + 3.8
1230	M	6	5	9 + 2.8
LD_{50} = 940 µl/kg (560 – 1473 µl/kg), Y = –5.96 + 3.68 log(X)				

F = female; M = male

Determination of the cumulation index (CI). The toxic effects of repeated oral treatments were studied by the method of Lim et al. (1961). The duration of their test was extended to 28 days. Repeated oral treatment means the administration of gradually increasing doses starting at 9% of the acute oral LD_{50} value and increased 1.5 times every 4 days (Table 2). All rats surviving the treatment were subjected to gross pathological examination. The subchronic LD_{50} was calculated by the method of Finney (1971) using probit analysis, and evaluated as described by Lehoczky et al. (1979) and Adamis et al. (1987).

Table 2

Effects of repeated oral doses of 2,4-D-amine on rats

	Days of study						
	1–4	5–8	9–12	13–16	17–20	21–24	25–28
% of acute oral LD_{50}	9.0	13.5	20.0	30.0	45.0	68.0	100
Dose, µl/kg/day	103	154	229	343	515	778	1145
Mortality in females	0/20	0/20	0/20	1/20	2/20	16/20	1/20
Mean body weight of females (g)	223	225	228	231	235	214	–
/control (g)	219	210	226	224	229	236	239/
Mortality in males	0/20	0/20	0/20	2/20	9/20	9/20	–
Mean body weight of males (g)	332	338	347	350	343	334	–
/control (g)	336	348	358	364	377	377	386/
Dose applied stepwise, µl/kg	412	618	916	1374	2060	3114	4580
Total exposure, µl/kg	412	1030	1946	3320	5380	8494	13074

Cumulation index (CI) = LD_{50} subacute/LD_{50} acute

$$\text{CI (females)} = \frac{1867.7}{1145.5} = 1.63; \quad \text{CI (males)} = \frac{1415.7}{940.0} = 1.51$$

Results and discussion

The data relevant to the determination of the acute oral LD_{50} are presented in Table 1.

Compared to the control, no significant differences could be demonstrated in body weight gains either in the acute oral LD_{50} studies or after repeated oral treatment. In both studies, the most important clinical signs included occasional muscle tremors, ruffled fur, asphyxia, and decreased motility. At necropsy, gas-filled intestines and mild pulmonary oedema were observed.

The cumulation index determined in the subchronic toxicity study using repeated oral treatments was 1.63 for females and 1.51 for males. A definite tolerance could be observed in both sexes.

These findings indicate that repeated oral treatment of experimental rats with 2,4-D-amine is capable of increasing the test animals' metabolizing capacity, thus accounting for the observed tolerance. The data obtained from these experi-

ments can serve as reference values in calculating the correct dose to be used in subchronic and chronic toxicity studies of 2,4-D-amine salts.

Acknowledgements

The authors thank Mrs. A. Németh, Mrs. M. Takács and Mrs. K. Temleitner for their valuable technical assistance. This study was sponsored by the National Research Fund (OTKA), project no. T 6351.

References

Adamis, Z., Szlobodnyik, J. and Karmos-Várszegi, M. (1987): The determination of the cumulative toxicity of permethrin in animal experiments (in Hungarian). Egészségtudomány **31,** 269–275.

Erne, K. (1966): Distribution and elimination of chlorinated phenoxyacetic acids in animals. Acta Vet. Scand. **7,** 240–248.

Finney, D. J. (1971): Probitanalyses. University Press, London-Cambridge. p. 118.

Heath, R. G., Spann, J. W., Hill, E. F. and Kreitzer, J. F. (1972): Comparative dietary toxicities of pesticides to birds. Spec. Sci. Rep., Washington D. C., Wildlife 152.28.

Hill, E. F. and Camardese, M. B. (1986): Lethal dietary toxicities of environmental contaminants and pesticides to Coturnix. Fish and Wildlife Technical Report (Washington, D. C.) **2,** 33–50.

Khanna, S. and Fang, S. C. (1966): Metabolism of C14 labelled 2,4-D acid in rats. J. Agr. Food Chem. **14,** 500–507.

Lehoczky, K., Szeberényi, M. J. and Tátrai, E. (1979): Studies on the local and systemic toxic effects of the herbicide propachlor (Satecid 50 WP) in experimental animals (in Hungarian). Egészségtudomány **23,** 89–96.

Lim, R. K. S., Rink, K. G., Glass, H. G. and Soaje-Echague, E. J. (1961): A method for the evaluation of cumulation and tolerance by the determination of acute and subchronic median effective doses. Arch. Int. Pharmacodyn. **130,** 336–353.

Rowe, V. K. and Hymas, T. A. (1954): Summary of toxicological information on 2,4-D and 2,4,5-T type herbicides. Am. J. Vet. Res. **15,** 622–630.

Way, J. M. (1969): Toxicity and hazards of man, domestic animals and wildlife from some commonly used auxin herbicides. Residue Reviews **29,** 37–72.

Acta Veterinaria Hungarica 43 (2–3), pp. 359–367 (1995)

EXPERIMENTAL FURAZOLIDONE TOXICOSIS IN BROILER CHICKS: EFFECT OF DOSAGE, DURATION AND AGE UPON CLINICAL SIGNS AND SOME BLOOD PARAMETERS

Q. ZAMAN[1], M. Z. KHAN[1], N. ISLAM[1] and G. MUHAMMAD[2]

[1]Department of Veterinary Pathology and [2]Department of Clinical Medicine and Surgery, Faculty of Veterinary Science, University of Agriculture, Faisalabad 38040, Pakistan

(Received August 17, 1994)

Groups of broiler chicks of one day and three weeks of age were given feeds containing 0, 400, 800 and 1000 mg furazolidone (Fz)/kg for three weeks. The age of the birds and the dose and duration of Fz treatment significantly ($P \leq 0.05$) influenced the parameters studied. Clinical signs of Fz toxicosis included ascites, leg weakness and nervous derangement like convulsions and torticollis. The body weight also decreased. Ascites and nervous derangement was not observed in birds fed 400 mg Fz from one day of age. Fz-fed birds developed anaemia and had lower plasma total protein and albumin levels than those fed no Fz. The decrease in body weight was related to reduced feed intake. Decreases found in the haematological parameters and plasma proteins showed no correlation with the chicks' age but were related to the dose and duration of Fz treatment.

Key words: Furazolidone toxicosis, broiler chicks, blood, plasma proteins

Furazolidone (Fz) is widely used in the prevention and treatment of certain bacterial and protozoal diseases of poultry. Due to a narrow margin between therapeutic and toxic levels it becomes toxic when administered for a prolonged duration or at levels higher than the therapeutic dose. Several reports have described the clinical and pathological picture in field cases of Fz toxicosis in broiler chickens (Wan and Yan, 1983; Orr et al., 1986; Sályi et al., 1986). Reports on experimental Fz toxicosis in White Leghorn chickens described growth retardation, anaemic changes and decrease in plasma proteins (Agate et al., 1983; Oyejide et al., 1983; Avramenko, 1985; Ali et al., 1988). Experimental Fz toxicosis in broiler chicks has resulted in retarded growth, ascites and cardiac biventricular dilatation (Reed and Van Vleet, 1988). However, in broiler chicks there is scanty information on the effect of age on the severity of clinical toxic reaction and on alterations in different blood parameters.

0236–6290/95/$ 4.00 © 1995 *Akadémiai Kiadó, Budapest*

With these considerations in mind, we report the effect of three dose levels of Fz, administered via the feed, on the clinical picture and different blood variables of broiler chicks of one day and three weeks of age.

Materials and methods

Experimental procedures

One hundred and sixty, day-old, broiler chicks (Hubbard) of both sexes were divided into eight equal groups and reared on rice husk. The basal feed given to these birds contained 21% total protein and no anticoccidial agent. Three other feeds were prepared from the basal feed by the addition of different levels of furazolidone (Furazole 24.4%, Hilton Pharma, Pakistan).

In the first experiment, four groups of one-day-old broiler chicks, designated as F0, F400, F800 and F1000, were given feeds containing 0, 400, 800 and 1000 mg Fz/kg, respectively.

In the second experiment, birds in the remaining four groups were kept on basal feed for three weeks and then were switched over to the Fz-supplemented feeds as in the first experiment. The duration of the experiments was four weeks.

Clinical evaluation

Birds were examined twice daily for the appearance of clinical signs and behavioural alterations. The feed intake of each group and the body weights of ten randomly selected birds from each group were recorded at weekly intervals.

Haematological and biochemical studies

About 2 ml of blood was drawn from the wing vein of six birds randomly selected from each group on days 14 and 28 of the experiment. Na_2EDTA was used as anticoagulant. A small aliquot of blood was used for the estimation of erythrocyte and total leukocyte number (Natt and Herrick, 1952), haematocrit (microhaematocrit method) and haemoglobin levels (cyanmethaemoglobin method). Plasma separated from the remaining blood was used for the estimation of total proteins and albumin by the Biuret method as described by Oser (1976).

Statistical analysis

The data were subjected to analysis of variance and different group means were compared by Duncan's multiple range test using a MStat-C statistical package on a personal computer. The probability level was 0.05 or lower.

Table 1

Clinical signs and mortality in different groups of broiler chicks fed furazolidone starting at one day (first experiment) and three weeks of age (second experiment)

Group[1]	First experiment			Second experiment		
	F400	F800	F1000	F400	F800	F1000
Clinical signs[2]						
Ascites						
No. of birds affected	0	3	3	2	4	4
Appeared on day[3]	–	12	10	17	15	16
Leg weakness						
No. of birds affected	1	1	1	1	0	3
Appeared on day	16	2	1	8	–	14
Nervous derangement (convulsions and torticollis)						
No. of birds affected	0	7	10	6	8	14
Appeared on day	–	8	7	18	14	8
Mortality (%)	10	25	45	15	35	55

n = 20 in each group in both experiments

[1]Groups F400, F800 and F1000 were given furazolidone for 4 weeks at rate of 400, 800 and 1000 mg/kg feed, respectively

[2]None of the birds in the control group (given feed without furazolidone) showed these clinical signs

[3]Days after treatment when sign first appeared

Results

Clinical signs

Clinical signs associated with furazolidone toxicosis in broiler chicks were depression, ascites, leg weakness and nervous derangements like torticollis, convulsions and aimless movements. A comparison of salient clinical signs between the two experiments is given in Table 1. In both experiments the number of birds showing torticollis and convulsions rose with the increase of dietary Fz level. Ascites, convulsions and torticollis were not observed in birds fed 400 mg Fz/kg in the first experiment. In older birds (fed Fz from 3 weeks of age in the second experiment) the clinical signs appeared relatively late and in a greater number of birds compared with the younger birds (those fed Fz from 1 day of age in the first experiment). Cumulative mortality in different groups of both experiments rose

parallel with the increase in dietary Fz level. A higher with birds of the first experiment, given the same levels of Fz.

Body weight gains

Fz at all dietary levels resulted in a decreased body weight gain (Table 2). During the first experiment, this decrease was nonsignificant in the first week for all groups; however, in the second experiment Group F1000 showed a significant decrease compared with the control. From the second week onward, all Fz-fed groups in both experiments had significantly lower body weights compared with the control. Reduced body weight gain occurred at increasing Fz levels of feed in both experiments but a significant difference between all treatment groups was present only in the first experiment at weeks 3 and 4 (Table 2).

Haematological and biochemical parameters

Different haematological parameters of Fz-fed groups of both experiments are presented in Table 3. A decrease in erythrocyte number was observed for all Fz-fed groups in both experiments. This significant decrease appeared at week 4 in the first experiment but at week 2 in the second experiment, compared with the control. Haematocrit values at week 2 were significantly lower than the control only in group F1000 of the first experiment. At week 4 haematocrit values were significantly lower than the control in all Fz-fed groups in both experiments. In the first experiment, haemoglobin levels were significantly lower in groups F800 and F1000 at week 4, whereas in the second experiment all Fz-fed groups had lower levels at week 2. In the second experiment, haemoglobin levels at week 4 were significantly lower in Groups F400 and F1000, but not in Group F800, compared with the control. Differences in leucocyte count were nonsignificant in all groups of both experiments (not shown in the table).

Total plasma protein levels decreased significantly in group F400 of both experiments at 2 weeks compared with the control (Table 4). At week 4, Groups F800 and F1000 of both experiments had significantly lower total plasma protein levels than the control. Albumin levels were significantly lower in Group F1000 in the first and in Groups F800 and F1000 in the second experiment at week 4. Globulin levels decreased significantly in group F400 in the second experiment at week 2. At week 4, the decrease in globulin level was significant in Groups F800 and F1000 in the first and in Group F1000 in the second experiment, as compared with the control.

Table 2

Body weight gains in different groups of broiler chicks fed furazolidone starting at one day (first experiment) and three weeks of age (second experiment) (mean g ± SD)

Group[1]	Body weight gain*				Feed consumption (g/bird)
	Week 1	Week 2	Week 3	Week 4	
			First experiment		
F0	38.5a ± 8.1	128.7a ± 23.5	272.7a ± 35.8	471.0a ± 49.3	1134.8
F400	38.8a ± 17.0	70.5b ± 17.4	194.9b ± 69.3	317.0b ± 76.7	763.6
F800	14.9a ± 10.1	69.2b ± 25.2	126.0c ± 63.7	206.9c ± 68.1	498.8
F1000	23.4a ± 11.5	44.3b ± 15.2	76.5d ± 26.6	145.2d ± 66.6	466.3
			Second experiment		
F0	205.0a ± 42.6	484.8a ± 209.0	779.0a ± 276.9	1144.0a ± 250.3	3243.6
F400	65.8ab ± 43.8	295.4b ± 231.5	567.7b ± 432.0	679.3b ± 191.6	2169.6
F800	78.5ab ± 64.9	266.1b ± 146.5	415.6bc ± 164.1	539.1b ± 119.4	1819.9
F1000	9.6b ± 65.1	199.4b ± 123.6	332.4c ± 119.7	503.4b ± 221.9	1463.7

n = 20 in each group of both experiments

* Body weight gain was calculated by subtracting the weekly weight of individual birds from the initial mean weight at day 0 which was 32 g in the first and 275 g in the second experiment.

[1] Groups F400, F800 and F1000 were given furazolidone for 4 weeks at a rate of 400, 800 and 1000 mg/kg feed, respectively.

Values in the same column with different letters are statistically significant ($P \leq 0.05$)

Table 3

Haematological parameters (mean ± SD) in different groups of broiler chicks fed furazolidone for four weeks starting from one day (first experiment) and three weeks (second experiment) of age

Groups[1]	Erythrocytes (10^6/µl)		Haemotocrit (%)		Haemoglobin (g/dl)	
	Week 2	Week 4	Week 2	Week 4	Week 2	Week 4
			First experiment			
F0	1.71a ± 0.46	1.84a ± 0.20	25.83a ± 1.32	25.33a ± 1.63	8.44a ± 0.60	8.73a ± 0.73
F400	1.65a ± 0.65	1.04b ± 0.26	25.50a ± 3.21	21.33b ± 5.28	8.09a ± 0.74	7.86a ± 1.38
F800	1.22a ± 0.36	1.21b ± 0.36	23.83 ab ± 1.94	21.00b ± 2.61	7.69a ± 1.56	5.99b ± 0.56
F1000	1.15a ± 0.47	1.07a ± 0.29	21.33b ± 2.73	19.83b ± 4.44	6.41b ± 0.65	6.30b ± 1.31
			Second experiment			
F0	2.07a ± 0.48	2.29a ± 0.19	26.83a ± 2.13	27.83a ± 1.17	10.57a ± 1.80	10.72a ± 2.60
F400	1.18b ± 0.24	1.41b ± 0.43	25.50a ± 0.63	23.33b ± 2.16	7.72b ± 0.46	8.58b ± 0.81
F800	1.32b ± 0.46	1.38b ± 0.38	26.42a ± 3.01	24.50b ± 2.59	7.72b ± 0.88	9.11ab ± 1.54
F1000	1.48b ± 0.40	1.33b ± 0.52	25.75a ± 4.58	25.00b ± 2.53	7.53b ± 0.97	8.94b ± 1.29

n = 20 in each group of both experiments

[1]Groups F0, F400, F800 and F1000 were given furazolidone for 4 weeks at a rate of 0, 400, 800 and 1000 mg/kg feed, respectively.

Values in each column with different letters are statistically significant ($P \leq 0.05$)

Table 4

Total plasma protein, albumin and globulin levels (mean ± SD) in different groups of broiler chicks fed furazolidone for four weeks starting from one day (first experiment) and three weeks (second experiment) of age

Groups[1]	Total protein (g/dl)		Albumin (g/dl)		Globulin (g/dl)	
	Week 2	Week 4	Week 2	Week 4	Week 2	Week 4
			First experiment			
F0	3.46a ± 0.63	3.59a ± 0.15	1.53a ± 0.14	1.56b ± 0.08	1.93a ± 0.71	2.03a ± 0.20
F400	2.93b ± 0.31	3.52ab ± 0.39	1.48a ± 0.13	1.87a ± 0.29	1.45a ± 0.35	1.66ab ± 0.28
F800	3.29ab ± 0.31	3.09b ± 0.56	1.45a ± 0.06	1.92a ± 0.32	1.84a ± 0.33	1.16b ± 0.50
F1000	3.11ab ± 0.23	3.03b ± 0.36	1.44a ± 0.08	1.64b ± 0.14	1.67a ± 0.21	1.19b ± 0.67
			Second experiment			
F0	4.40a ± 0.89	3.59a ± 0.69	1.43a ± 0.19	1.64a ± 0.10	2.97a ± 0.88	1.93a ± 0.68
F400	3.54b ± 0.64	3.69a ± 0.46	1.41a ± 0.05	1.65a ± 0.10	2.14b ± 0.66	2.15a ± 0.25
F800	4.62a ± 0.53	3.15b ± 0.59	1.44a ± 0.06	1.51b ± 0.02	3.16a ± 0.52	1.68ab ± 0.57
F1000	4.38a ± 0.47	2.45b ± 0.44	1.42a ± 0.05	1.53b ± 0.04	2.96a ± 0.49	1.14b ± 0.40

n = 20 in each group of both experiments

[1]Groups F0, F400, F800 and F1000 were given furazolidone for 4 weeks at a rate of 0, 400, 800 and 1000 mg/kg feed, respectively.

Values in each column with different letters are statistically significant ($P \leq 0.05$)

Discussion

Clinical signs of Fz toxicosis observed in the present experimental study were similar to those reported in broiler chickens and other avian species (Van Vleet and Ferrans, 1983; Orr et al., 1986; Sályi et al., 1986; Reed et al., 1980; Webb and Van Vleet, 1991). For chicken the therapeutic level of Fz in feed is 0.04% for 10 days (Brander et al., 1982). The present study showed that the prolonged used of therapeutic levels of Fz produced more severe clinical signs and higher mortality in older birds (second experiment) compared with young birds fed the same level of Fz in the first experiment. This observation suggests that the severity of Fz toxicosis increases with age. A dose-related increase in the number of clinically sick birds has been reported (Reed and Van Vleet, 1988), but an age-related increase in the severity of Fz toxicosis as observed in the present study has not been reported thus far. In the present study, the absence of clinical signs, the low mortality and the nonsignificant decrease in body weight seen in the first experiment among birds fed 400 mg Fz/kg feed suggested that in field situations and in young broiler chicks clinical toxicosis caused by low levels of Fz may not be detectable until the drug is administered continuously over a prolonged period. Orr et al. (1986) reported a mortality and culling rate of 15–16.6 per cent in two broiler flocks fed 0.033 per cent Fz for over 3 weeks; however, in another flock the same Fz level fed from 1 to 17 days of age produced only slight effects. These workers assumed that drug toxicity might have been exacerbated by other additives like NaCl, monensin and amprolium. However, in addition, the young age of the birds might also be responsible for minor losses. Similarly, Sályi et al. (1986) could not induce clinical Fz toxicosis by feeding 300 mg Fz/kg from one day of age, but disease was produced by feeding 900 mg Fz/kg. The absence of clinical disease in birds fed 400 mg Fz/kg for four weeks from one day of age (first experiment) also suggests that this dose and age of the birds might not be suitable for the experimental production of clinical Fz toxicosis.

The deterioration of the haematological parameters of Fz-intoxicated birds in both experiments indicated the anaemia-producing character of Fz, which has been scarcely investigated in broiler chicks. Avramenko (1985) reported reduced haemoglobin levels in 5-month-old White Leghorn chickens given a single dose of 10 mg Fz/kg body weight. Ali et al. (1988) reported a decrease in haematological parameters in 3-month-old White Leghorn chickens three days after administration of a single toxic dose of 200 mg Fz/kg body weight. No information is available about the effects of Fz upon haematological parameters in broiler chicks. The present findings suggested that the development of anaemic changes was related neither to the concentration of Fz in the feed ($\geq$ 400 mg Fz/kg) nor to age of the birds but depended upon the duration of treatment. Whether anaemic changes resulted from any direct toxic effects of Fz upon haemopoietic tissue or occurred as a generalised weakness due to the anorexic condition is not known. Decreases in

the plasma proteins, albumin and globulin have been reported in White Leghorn (Agate et al., 1983; Oyejide et al., 1983; Ali et al., 1988) but not in broiler chicks. The present study revealed that, like anaemic changes, the decrease in total protein, albumin and globulin was not related to the age of the birds and occurred parallel to the increase in dietary Fz level. The decrease in the body weight of broiler chicks in the present study depended on the level of Fz in the feed and was associated with a decreased feed intake. However, the dose-dependent decrease in body weight in groups fed different levels of Fz was significant only in the first experiment. Decrease in the body weight could be attributed to the anorexic conditions that developed during Fz toxicosis. The anorexigenic potential of Fz, reported earlier (Ali and Bartlet, 1982), was also evident in the present study, as indicated by a nonsignificant difference in the feed conversion ratios in all groups despite the significant decrease in body weight.

In conclusion, the present study describes the clinical signs and the haematological as well as biochemical changes induced by Fz in broiler chicks. Nervous derangements did not appear in chicks fed 400 mg Fz/kg for 4 weeks from 1 day of age. All the parameters studied were significantly affected by the age of the birds and the dose or duration of Fz treatment.

References

Agate, A., Ehofor, M. I. and Sokale, E. A. (1983): The effects of furazolidone administration on body weight and serum biochemistry of chickens. Bull. Anim. Health Prod. Africa **31,** 269–273.

Ali, B. H. and Bartlet, A. L. (1982): Anorexia and antagonism of thiamine utilization in poultry treated with furazolidone. Quart. J. Exp. Physiol. **67,** 437–448.

Ali, B. H., Idris, O. F., Fadalla, A. M., Awad, A. H., Karim, E. L. and Aradaib, I. E. (1988): Some effects of furazolidone and furaltadone in chickens. Eur. Assoc. Pharmacol. Toxicol. Budapest, Hungary 28 August–2 September 1988 (Abstract).

Avramenko, N. V. (1985): Haemoglobin and iron in the blood of fowls given furazolidone. Veterinariya (Moscow) **7,** 59–60.

Brander, G. C., Pugh, D. M. and Bywater, R. J. (1982): Veterinary Applied Pharmacology and Therapeutics. Bailliere Tindall, London. 432 pp.

Natt, M. P. and Herrick, C. A. (1952): A new diluent for counting the erythrocytes and leukocytes of the duck. Poult. Sci. **31,** 735–737.

Orr, J. P., Little, K. S., Schoonderwoerd, M. and Rehmtulla, A. J. (1986): Ascites in broiler chickens. Can. Vet. J. **27,** 99–100.

Oser, B. L. (1976): Hawk's Physiological Chemistry. Tata MaGraw Hill Pub. Co. New Delhi.

Oyejide, A., Ejiofor, M. I. and Sokale, E. A. (1983): The effects of furazolidone administration on body weight and serum biochemistry of chickens. Bull. Anim. Health Prod. Africa **31,** 269–273.

Reed, W. M., Van Vleet, J. F., Wigle, W. L. and Fulton, R. M. (1980): Furazolidone associated cardiomyopathy in two Indiana flocks of ducklings. Avian Dis. **31,** 666–672.

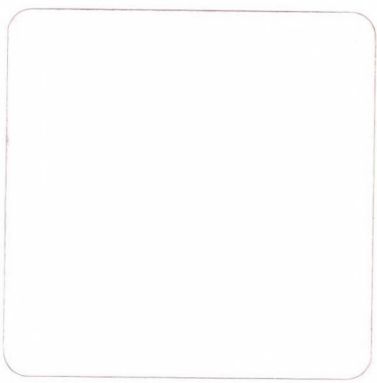
Reed, W. M. and Van Vleet, J. F. (1988): Furazolidone-induced cardiomyopathy and ascites in broilers. Proc. West. Poult. Dis. Conf. **37,** 156–160.

Sályi, G., Povazsán, J. Ivanics, E., Glávits, R. and Bagó, Gy. (1986): Furazolidone poisoning in a large broiler flock (in Hungarian, with English abstract). Magyar Állatorvosok Lapja **41,** 407–415.

Van Vleet, J. F. and Ferrans, V. J. (1983): Congestive cardiomyopathy induced in ducklings fed graded amounts of furazolidone. Am. J. Vet. Res. **44,** 76–85.

Wan, X. and Yan, E. (1983): Furazolidone poisoning in chicken. Chinese J. Vet. Med. **9,** 29–30.

Webb, D. M. and Van Vleet, J. F. (1991): Early clinical and morphologic alterations in the pathogenesis of furazolidone induced toxicosis in ducklings. Am. J. Vet. Res. **52,** 1531–1536.



PRINTED IN HUNGARY

Akadémiai Kiadó és Nyomda, Budapest

MAGYAR
TUDOMÁNYOS AKADÉMIA
KÖNYVTÁRA

INSTRUCTIONS TO AUTHORS

Manuscripts are accepted on the basis of scientific significance and suitability for publication on the understanding that they have not been published, submitted or accepted for publication elsewhere. Acceptance depends on the opinion of two referees and the decision of the Editorial Board. Papers accepted for publication are subject to editorial revision. Only original papers will be published and a copy of the Publishing Agreement will be sent to the authors of papers accepted for publication. Manuscripts will be processed only after receiving the signed copy of the agreement.

MANUSCRIPT STYLE

Manuscripts must be in English or Hungarian and clearly and concisely written. They should be typed double spaced with wide margins. Two copies of the manuscript should be submitted.

FORM OF MANUSCRIPT

Title. The title should be a clear and concise statement of the contents in not more than 14 words. A short running title of not more than 50 letters should also be supplied. This is followed by the authors' initials (full first name of women) and surname, and the name of the institution where the work was done. The mailing address of the authors must also be indicated here.

Abstract. This should not exceed 200 words and should outline briefly the purpose of the study and detail important findings and the authors' principal conclusions. Redundant phrases, generally known information and repetition should be avoided. Four or five keywords should also be supplied.

Introduction. This part should state briefly the nature and purpose of the work and cite recent important work by others.

Materials and methods. Describe materials, methods, apparatus, experimental procedure and statistical methods in sufficient detail to allow other authors to reproduce the results. This part may have subheadings. The experimental methods and treatments applied shall conform to the most recent guidelines on the humane treatment and care of animals.

Results. The experimental data should be represented clearly and concisely. Avoid repeating information presented in tables and figures.

Discussion should be focussed on the interpretation of experimental findings. Do not repeat literary data presented in the Introduction or information given in Results. References should be cited as follows: e.g. ... as observed by Faith and Trump (1976); or in parentheses: ... were found (Starr et al., 1978; Manson and Starr, 1979).

Acknowledgement of grants and technical help.

References. Cite only essential references. They should be arranged in alphabetical order of the authors' surnames, without serial numbers. The reference list at the end of the paper should contain

- for journals: names and initials of all authors, year of publication (in parentheses), colon, English title of the paper (if the original title is not English, indicate in parentheses, e.g. [in French]), journal title abbreviated according to the style used in Index Veterinarius, volume number, issue number in parentheses, first and last pages;
- for books: names and initials of authors/editors, year (in parentheses), title, publisher, place of publication, page number.

Tables. They should be typed on separate sheets and have a concise heading each. Tables are to be numbered sequentially by Roman numbers.

Figures. Good-quality glossy prints a half-tone illustrations and clear line drawings in Indian ink are accepted. Colour photographs will be accepted, but the extra cost of reproduction in colour must be borne by the authors (in 1995 US$ 260 per page). The number of the figure, the author's name and the top of the figure should be indicated lightly in soft pencil on the back. The figures are to be numbered sequentially by Arabic numbers. Captions should be provided on a separate sheet. All figures should be referred to in the text and their approximate place indicated on the margin.

Abbreviations and symbols. Quantities should be expressed in SI units. All abbreviations should be spelled out when first used in the text. Please identify unusual symbols in the margin.

Proofs and reprints. One set of proofs will be provided, which is requested to be returned within 48 hours of receipt to the Editor. Alterations in the text and especially in the illustrations are expensive and should be avoided. Fifty reprints of each paper are supplied free of charge. Additional reprints can be ordered at cost price at the time the page proof is returned.

307209

9

Acta Veterinaria Hungarica

20.

VOLUME 43, NUMBER 4, 1995

EDITOR–IN–CHIEF

J. MÉSZÁROS

ASSOCIATE EDITOR

A. SZÉKELY

EDITORIAL BOARD

A. BARTHA, J. BOKORI, T. KASSAI, F. KUTAS,
G. PETHES, P. RUDAS, L. SOLTI, S. TUBOLY,
J. VARGA, F. VETÉSI. K. VÖRÖS

Akadémiai Kiadó, Budapest

ACTA VET. HUNG. 43 (4) 369–477 (1995) HU ISSN 0236–6290

ACTA VETERINARIA

A QUARTERLY OF THE HUNGARIAN ACADEMY OF SCIENCES

Acta Veterinaria publishes reviews and original papers on veterinary science in English.

Acta Veterinaria is published in yearly volumes of four issues by

AKADÉMIAI KIADÓ

Publishing House of the Hungarian Academy of Sciences

H–1117 Budapest, Prielle Kornélia u. 19–35

Manuscripts and editorial correspondence should be addressed to

Acta Veterinaria

Veterinary Medical Research Institute of the
Hungarian Academy of Sciences

H–1581 Budapest, P.O. Box 18

Subscription information

Orders should be addressed to

AKADÉMIAI KIADÓ

H–1519 Budapest, P.O. Box 245

Subscription price for Volume 43 (1995) in 4 issues US$ 96, including normal postage, airmail delivery US$ 20.00.

Acta Veterinaria is indexed in Current Contents

Acta Veterinaria Hungarica is abstracted/indexed in AGRICOLA, Biological Abstracts, Bibliography of Agriculture, Chemical Abstracts, Current Awareness in Biological Sciences, Current Contents–Agriculture, Biology and Environmental Sciences, Dairy Science Abstracts, Excerpta Medica, Focus On: Veterinary Science and Medicine, Helminthological Abstracts, Index Medicus, Index Veterinarius, International Bibliography of Periodical Literature, La Presse Médicale, Nutrition Abstracts and Reviews, Review of Plant Pathology, Soils and Fertilizers, Veterinary Bulletin.

"This periodical is included in the document delivery program THE GENUINE ARTICLE of the Institute of Scientific Information, Philadelphia. The articles published in the periodical are available through *The Genuine Article* at the Institute for Scientific Information, 3501 Market Street, Philadelphia PA 19104."

© Akadémiai Kiadó, Budapest 1995

CONTENTS

Animal reproduction

Successful embryo recovery from swine by a minimal invasive technique. *Rátky, J.* and *Brüssow, K.-P.* 371

Epizootiology

Achievements and difficulties in maintaining the tuberculosis-free status of Hungarian cattle herds. *Körmendy, B.* 377

The value of immunodiagnostic tests in detecting tuberculosis in an infected red deer herd and in eradication of the disease by selection. *Zomborszky, Z., Körmendy, B., Tuboly, S., Tilly, P.* and *Horn, P.* 385

Mycotoxin research

Detection of ochratoxin A in human blood and colostrum. *Kovács, F., Sándor, Gabriella, Ványi, A., Domány, S.* and *Zomborszky-Kovács, Melinda* 393

Parasitology

Dynamics of *Anguillicola crassus* (Nematoda: Dracunculoidea) larval infection in paratenic host fishes of Lake Balaton, Hungary. *Székely, Cs.* 401

Pathophysiology

Evaluation of blood lipid peroxidation parameters in carbon tetrachloride (CCl_4) toxicity in sheep. *Vajdovich, P., Szilágyi, A.* and *Gaál, T.* 423

Effects of histamine on gizzard erosions and on the activity of selected enzymes in chickens. *Džaja, P., Grabarević, Ž., Perić, J., Artuković, Branka, Tišljar, Marina, Mrljak, V.* and *Šoštarić, Edita* 431

Physiology

Differences in the thryroxine, triiodothyronine and reverse triiodothyronine contents of fetal pig tissues relative to gestational age. *Krysin, Ewa* 443

Seasonal variation in the concentration of vitamins A and E in the blood plasma of fat-tailed sheep. *Asadian, S., Mirhadi, S. A.* and *Mézes, M.* 453

Variations in the yield, composition and somatic cell count of ewe's milk during lactation. *Bedő, S., Nikodémusz, Etelka* and *Gundel, Katalin* 463

Coming Event 475

Announcement 477

MAGYAR
TUDOMÁNYOS AKADÉMIA
KÖNYVTÁRA

Acta Veterinaria Hungarica 43 (4), pp. 371–375 (1995)

SUCCESSFUL EMBRYO RECOVERY FROM SWINE BY A MINIMAL INVASIVE TECHNIQUE

J. RÁTKY[1] and K.-P. BRÜSSOW[2]

[1]Research Institute for Animal Breeding and Nutrition, H–2053 Herceghalom, Hungary; [2]Research Institute for the Biology of Farm Animals, 18196 Dummerstorf, Wilhelm-Stahl-Allee 2, Germany

(Received October 2, 1995)

An endoscopic embryo flushing technique was elaborated to make possible the minimal invasive collection of ova from swine. After experience with pig laparoscopy, the method of endoscopic embryo recovery in small ruminants was adapted to the abdominal anatomy of swine. Twelve oestrus-synchronized gilts were operated 5 days after artificial insemination. In 10 cases both uterine horns were flushed by using a Rüsch-Gold balloon catheter, flushing cannula and atraumatic grasping forceps beside the 10 mm diameter optic. The whole procedure took approximately 35–45 min. The average collection rate was 40.4%. According to the results, endoscopic embryo recovery can be a useful method in swine reproductive research and in top breeding in the future.

Key words: Endoscopy, embryo collection, swine

Although abdominal endoscopy is widely used in human surgery (e.g. in gynaecology) and is well known in the reproductive research of ruminants, there are few data in the literature on the use of this minimal invasive technique in swine. The existing data concern the adaptation of laparoscopy to swine (Wildt et al., 1973; Dukelow, 1978; Brüssow et al., 1990), the diagnosis of pathological features (Wekerle et al., 1990; Rátky, 1993), or observation of different treatment effects on reproductive events (Brüssow and Rátky, 1993; Brüssow et al., 1993).

Endoscopic gynaecological surgery has been used for several purposes in ruminants: for inseminating ewes and goats (Cseh et al., 1986; McKelley and Robinson, 1986; Fièni et al., 1991), for embryo transfer in ewes (Walker et al., 1985), and for the collection of embryos from sheep (McKelley and Robinson, 1986; Scudamore et al., 1991). Sirard and Lambert (1986) aspirated oocytes by endoscopy for *in vitro* fertilization, and this was repeated later in calves (Amstrong et al., 1991).

Information on similar procedures in swine can hardly be found, due to the anatomy of the genital organs which is less convenient to be operated, and to the multiparity of this species. Wildt et al. (1975) reported on uterine fluid collection to analyze the content on different days of pregnancy. Nearly 20 years later, re-

0236–6290/95/$ 5.00 © 1995 *Akadémiai Kiadó, Budapest*

peated ovum pick-up and follicular fluid collection were published to study the *in vivo* maturation of pig oocytes and to establish a method for producing ova from live gilts (Brüssow and Rátky, 1994).

Although embryo transfer is not as important in swine as in uniparous species such as ruminants, it can be useful in top breeding and research. There have been some attempts to elaborate nonsurgical embryo flushing methods for gilts (Altenhof et al., 1982; Hazeleger et al., 1989), but actually the only way to collect porcine embryos is by surgery. However, when Stein-Stefanie and Holtz (1986) compared the laparotomic and laparoscopic transfer in pigs, they did not carry out endoscopic embryo collection. Since in the last decade more than 1,500 gilts and sows were laparoscopized by our research group, the aim of the present study was to elaborate an endoscopic embryo flushing method for pigs, following a brief communication presented on this topic (Rátky and Brüssow, 1995).

Materials and methods

Twelve puberal crossbred Landrace gilts of 7 months of age and weighing 90–100 kg were synchronized by per os administration of altrenogest (Regumate, Roussel Uclaf) 5 g daily for 15 days. On day 16, gilts were injected with 1000 IU PMSG (Pregmagon, Dessau) followed by 50 μg GnRH (Gonavet, Veyk-Pharma) 80 h later. On the subsequent day the gilts were inseminated twice with commercial doses of semen. Five days after insemination the animals were subjected to surgery.

Twenty-four h prior to surgery the feed and water were withdrawn. Gilts were anaesthetized by iv. administration of 5 ml xylazine (Xilavet, Lavet) and 15 ml ketamine (Calypsovet, Gedeon Richter) and fixed on an operating table in Trendelenburg's position. Following disinfection of the abdominal wall the abdominal cavity was filled with CO_2 to achieve pneumoperitoneum which is essential for visualizing the abdominal organs. One 10 mm diameter and four 5 mm trocars were introduced into the abdomen for the optic and the grasping forceps, respectively. The 10 mm trocar was placed 2–3 cm caudal to the umbilicus, the first 5 mm trocar was 10 cm caudal to the 10 mm trocar in the linea alba, and the second 5 mm trocar between the last paps. Two 5 mm trocars were placed 4 cm bilateral to the first one. The setup of instruments was described earlier (Brüssow et al., 1990).

The ovarian structures were evaluated according to Schnurrbusch et al. (1981) mainly to check the previous ovulations by the presence of 5-day old corpora lutea. A Rüsch-Gold balloon catheter (Ch.12) was inserted into the uterine lumen cc. 15 cm caudal to the uterotubal junction and the balloon was inflated. A 30 cm long 16G aspiration cannula was introduced into the uterus right caudal to the junction where the uterine horn was fixed with a grasping forceps.

The uterine horn was filled and flushed with 70 ml PBS via aspiration cannula and Rüsch-catheter. Then the Rüsch-catheter and the cannula were pulled out and the procedure was repeated in the contralateral uterine horn. If myometrial prolapse was suspected, the wound was sutured endoscopically, using an intracorporal knot. After the completion of embryo collection all instruments were removed and the five 1–2 cm long incisions on the skin sutured.

Results

A total of 20 uterine horns of 10 gilts were flushed successfully. The results of embryo recovery are demonstrated in Table 1.

Table 1

Results of endoscopic embryo collection

Number of gilts	n	12
Number of flushed uterine horns	n	20
Success rate of flushing	%	83
Number of corpora lutea	n	146
Number of collected ova	n	59
Recovery rate	%	40.4

The recovery rate varied between 0% and 100%. Flushing fluid was recovered totally in 17 cases. Endoscopic embryo collection from both uterine horns took about 35–45 min. The volume of the flushing medium was measured and the ova isolated and counted microscopically.

Pigs were transported back to their shed. No post-operative complication was detected in the following two weeks.

Discussion

Apart from a short report (Brüssow and Rátky, 1994), the veterinary laparoscopic literature contains no data on successful endoscopic embryo recovery in swine. Therefore, the aim of this study was to establish a minimal invasive embryo collection method in pigs. The basis for this work was constituted by the more than 1500 diagnostic laparoscopies carried out in the last ten years and the endoscopic ovum pick-up elaborated for swine two years ago (Brüssow and Rátky, 1994). Hitherto only Stein-Stefanie and Holtz (1986) have reported on the endoscopic transfer of swine embryos; however, they did not use laparoscopy for

embryo recovery. The endoscopic embryo recovery method used in sheep (McKelley and Robinson, 1986) was adapted to pigs, but in pigs a two-way Rüsch-catheter was used and the uterine horn was flushed through unlike the technique in sheep. Since the uterotubal junction and the ipsilateral uterine horn had to be fixed, a five-point laparoscopy was elaborated, i.e. one 10 mm trocar and four 5 mm trocars were used for the optic and for the atraumatic grasping forceps, flushing cannula and Rüsch-catheter, respectively.

Although the achieved recovery rate is not satisfactory yet, embryo collection by this minimal invasive method is possible. Further experiments are needed to achieve a higher collection rate and to prove that the technique can be repeated at short intervals in the same animals, since this procedure can be a valuable technique in swine reproductive research and in top breeding.

References

Altenhof, R. L., Tanksley Jr., T. D., Knabe, D. A., Harms, P. G., Bowen, M. J. and Kraemer, D. C. (1982): Investigations on nonsurgical embryo collection in swine. Theriogenology **17,** 75.

Amstrong, D. T., Holm, P., Irvine, B., Petersen, B. A., Stubbings, R. B., McLean, D., Stevens, G. F. and Seamark, R. F. (1991): Laparoscopic aspiration and *in vitro* maturation of oocytes from calves. Theriogenology **35,** 182.

Brüssow, K.-P. and Rátky, J. (1993): The duration of ovulation in GnRH treated gilts following seminal plasma infusion. Reprod. Dom. Anim. **28,** 119–122.

Brüssow, K.-P. and Rátky, J. (1994): Repeated laparoscopical puncture and oocyte aspiration in swine. Reprod. Dom. Anim. **29,** 494–502.

Brüssow, K.-P., Rátky, J. and Kanitz, E. (1993): The influence of exogenous GnRH on the time of ovulation in gilts — an endocrine and laparoscopic study. Arch. Tierz. **36,** 197–203.

Brüssow, K.-P., Rátky, J., Kanitz, W. and Becker, F. (1990): Determination of the duration of ovulation in gilts by means of laparoscopy. Reprod. Dom. Anim. **25,** 184–190.

Cseh, S., Bilton, R. J. and Bényei, B. (1986): Fertilization of donor ewes by laparoscopy after superovulatory treatment (in Hungarian, with English abstract). Magyar Állatorvosok Lapja **41,** 55–57.

Dukelow, W. R. (1978): Laparoscopic research techniques in mammalian embryology. In: Dukelow, W. R. (ed.) Methods in Mammalian Reproduction. Academic Press, New York, 1978.

Fièni, F., Buggin, M., Tainturier, D., Bach-Lijour, B., Bruyas, J. F. and Daubie, M. (1991): Study of the best hour for intrauterine insemination in young dairy goats after hormonal induction of estrus. Theriogenology **35,** 200.

Hazeleger, W., van der Meulen, J. and van der Lende, T. (1989): A method for transcervical embryo collection in the pig. Theriogenology **32,** 727–734.

McKelley, W. A. C. and Robinson, J. J. (1986): Embryo survival and growth in the ewe — Recent studies on the effects of nutrition and on novel techniques for the recovery and transfer of embryos. A. Rep. Studies in Anim. Nutrit. and Allied Sci. of the Rowett Res. Inst. **41,** 9–25.

Rátky, J. (1993): Endoskopischen Eierstocksuntersuchungen bei Anöstrischen Jungsauen. Reprod. Dom. Anim. Suppl. **2,** 165.

Rátky, J. and Brüssow, K.-P. (1995): Pelviscopical methods in swine reproductive research. International Workshop on "Advanced Gynaecological Endoscopic Surgery and Vaginal Surgery". Bordeaux, France, 15–16 September, 1995, p. 52.

Schnurrbusch, U., Bergfeld, J., Brüssow, K.-P. and Kaltofen, U. (1981): Schema zur Ovarbeurteilung beim Schwein. Mh. Vet. Med. **36,** 811–815.

Scudamore, G. L., Robinson, J. J., Aitken, R. P., Kennedy, D. J., Ireland, S. and Robertson, I. S. (1991): Laparoscopy for intrauterine insemination and embryo recovery in superovulated ewes at a commercial embryo transfer unit. Theriogenology **35,** 329–337.

Sirard, M. A. and Lambert, R. D. (1986): Birth of calves after *in vitro* fertilization using laparoscopy and rabbit oviduct incubation of zygotes. Vet. Rec. **119,** 167–169.

Stein-Stefanie, J. and Holtz, W. (1986): Embryotransfer beim Schwein mit Hilfe der Endoskopie. Zuchthygiene **4,** 176.

Walker, S. K., Warnes, G. M., Quinn, P., Seamark, R. F. and Smith, D. H. (1985): Laparoscopic technique for the transfer of embryos in sheep. Austral. Vet. J. **62,** 105–106.

Wekerle, L., Rátky, J. and Sebestyén, J. (1990): Ovarian findings of gilts judged anoestrous by clinical examination (in Hungarian, with English abstract). Magyar Állatorvosok Lapja **45,** 269–273.

Wildt, D. E., Fujimoto, S., Spencer, J. L. and Dukelow, W. R. (1973): Direct ovarian observation in the pig by means of laparoscopy. J. Reprod. Fert. **35,** 541–543.

Wildt, D. E., Morcom, G. B. and Dukelow, W. R. (1975): Laparoscopic pregnancy diagnosis and uterine fluid recovery in swine. J. Reprod. Fert. **44,** 301–304.

Acta Veterinaria Hungarica 43 (4), pp. 377–384 (1995)

ACHIEVEMENTS AND DIFFICULTIES IN MAINTAINING THE TUBERCULOSIS-FREE STATUS OF HUNGARIAN CATTLE HERDS

B. KÖRMENDY

Central Veterinary Institute, H–1149 Budapest XIV, Tábornok u. 2, Hungary

(Received January 9, 1995)

The tuberculosis-free status of Hungarian cattle herds in the period between 1988 and the end of 1993 is evaluated. An epidemiological analysis of tuberculin tests, laboratory assays and allergic tests yielding positive results, summarized in three tables, is given with respect to the cattle population of Hungary. The origin of positive reactions obtained in the tuberculin tests was traced in different farms of a total of 323 communities. On those farms, the diagnostic slaughter and examination of 1,851 breeding animals to exclude or confirm *Mycobacterium bovis* infections involved substantial economic losses. From 25 outbreaks investigated in the period of study, a total of 191 *M. bovis* strains were isolated from the organs of 21 cattle in 15 household stocks in 14 communities, as well as from 170 bovine organ samples from large farms of 10 communities (7 agricultural co-operatives and 3 state farms). In all of these cases, infection could be traced back to humans excreting *M. bovis*. Determination of the 2-thiophenecarboxylic hydrazide (TCH) resistance of the isolates facilitated the epidemiological investigation. The paper also contains some recommendations on the prevention or reduction of losses.

Key words: Bovine tuberculosis, *Mycobacterium bovis*, incidence, Hungary

The eradication of tuberculosis from Hungarian cattle herds was completed in 1980 (Dénes, 1983*a, b, c*). The number of cows free from tuberculosis exceeded 600,000. Some of the herds were kept under loose housing conditions while others in the tie-up system. The individual and herd marking of cattle was an essential requirement for the eradication and serves as the basis of the current testing system.

The eradication of tuberculosis from cattle herds meant exclusively the elimination of *Mycobacterium bovis* infection.

Different mycobacterial infections of cattle may give rise to reactions of dissimilar type (Lepper and Corner, 1983). While *M. bovis* and *M. paratuberculosis* can produce visible lesions in several organs in addition to the local changes, *M. tuberculosis, M. avium, M. chelonei, M. farcinogenes, M. fortuitum, M. intracellulare, M. kansasi* and *M. scrofulaceum* can give rise to no other changes

0236–6290/95/$ 5.00 © 1995 *Akadémiai Kiadó, Budapest*

but local lesions. Other mycobacteria (*M. xenopi, M. microti, M. marinum*, etc.), nocardiae and sphagnums produce only seroconversion but no gross lesions in bovine animals (Lepper and Corner, 1983).

Under the conditions outlined above, the most important task of the animal health service is to protect the cattle herds from exposure to *M. bovis*-infected humans and animals of different species, to trace the origin of positive tuberculin reactions revealed during the annual tests, and to eliminate the consequences of possible *M. bovis* infections with the lowest economic losses.

The present paper analyzes the epizootiological experience and diagnostic results obtained during the past 6 years by the Department of Bacteriology and Mycobacteriology, the unit responsible for the diagnosis of tuberculosis at the Central Veterinary Institute of Hungary.

Materials and methods

Cattle herds must be checked for freedom from tuberculosis by annual tuberculin testing (Collection of New Animal Health and Food Hygienic Regulations, 1982).

Materials

(1) During the past decade, nearly 2,000,000 tuberculin tests were performed each year on cattle. These tests involved a total of 5,000 cattle farms and household stocks.

(2) In addition, about 400,000 cattle (fattening cattle, calves, culled cows, heifers) are subjected to post-mortem inspection by the Meat Inspection Service at abattoirs either within or outside Hungary. Post-mortem inspection includes a check for lesions caused by tuberculosis. If any lesions due to tuberculosis are detected, samples from the pathologically altered organs are submitted to the laboratory designated for mycobacteriological examinations in the given country. These samples are accompanied by a form containing all relevant data of the animal showing lesions suggestive of tuberculosis (country, county, farm, individual) and by the post-mortem report. In Hungary, these examinations are performed by the Department of Bacteriology and Mycobacteriology of the Central Veterinary Institute.

(3) On the occasion of the annual obligatory tuberculin testing, the veterinary authorities recommended or ordered the slaughtering and examination for tuberculosis of different numbers of cattle every year. In the period of this study, organ samples from a total of 1,848 cattle (402 in 1988, 215 in 1989, 283 in 1990, 264 in 1991, 349 in 1992, and 335 in 1993) were examined for tuberculosis.

Methods

(1) Epizootiological investigation of the herds includes checks of the management system, the records kept of individual animals and herds, the results of post-mortem examinations at slaughterhouses, the movements of animals, and the health records of the personnel dealing with the given herd.

(2) Intradermal tuberculin tests are carried out using bovine tuberculin (10,000 IU/dose) of PPD quality, produced from *M. bovis* strain AN5.

(3a) If a positive reaction is obtained, herd testing must be repeated 90 days later using bovine and avian allergen and paratuberculin to detect any allergenic bacteria (Collection of New Animal Health and Food Hygienic Regulations, 1982).

(3b) In order to judge the allergic reactions, blood samples from positive reactors are subjected to complement fixation and gel diffusion tests to detect paratuberculosis, and to a gel diffusion test using an antigen prepared from *M. avium* strain 16909-3380 (Körmendy et al., 1984).

(3c) Faecal samples from the reactors are tested for *M. paratuberculosis* by microscopy and by culturing (Körmendy, 1994).

(4) Samples taken from the predilective organs (tonsils, retropharyngeal, peribronchial and mediastinal lymph nodes, lungs, lymph node belonging to the ileocaecal valve, ileum) of animals giving a positive reaction in the allergic test, as well as those from diagnostically slaughtered animals are subjected to gross pathological, histopathological and bacteriological examination complemented with the inoculation of experimental animals. The bacteriological examinations are complemented with biochemical, drug sensitivity (Tsukamura, 1975) and DNA-DNA hybridization (Epignost GmbH, Linz) tests.

Results

The annual tuberculin tests performed between 1988 and 1993 using the intradermal method gave positive reactions in 5–15% of the cattle herds tested. The positivity rate varied with county. The ratio of positive reactors exceeded 1% in each positive herd. At the time of evaluating the intradermal tuberculin tests, the factors and the microorganism responsible for the allergic reaction are usually still unclear, although these factors and their incidence markedly affect the judgement formed of a given herd. While investigating the origin of the allergic reactions, the veterinary authorities should impose certain restrictions on husbandry and trade (prohibition of the movement of animals between groups and barns, organization of health checks for the personnel, delaying of the sale of breeding animals, pecuniary compensation paid for the milk, etc.), without imposing movement restrictions (Collection of New Animal Health and Food Hygienic Regulations, 1982).

Table 1

The incidence of positive tuberculin reactions and *Mycobacterium bovis* infections

County	Years																	
	1988			1989			1990			1991			1992			1993		
	Villages	Cows	*M. bovis* strain*	Villages	Cows	*M. bovis* strain*	Villages	Cows	*M. bovis* strain*	Villages	Cows	*M. bovis* strain*	Villages	Cows	*M. bovis* strain*	Villages	Cows	*M. bovis* strain*
Baranya	8	101	1(1I)	12	30	–	8	67	–	11	34	1(1I)	15	107	1(1I)	13	112	17(2)
Bács-Kiskun	5	27	–	–	–	–	1	1	–	–	–	–	1	2	–	–	–	–
Békés	6	28	–	5	9	1(1I)	3	5	–	9	65	16(1)	5	22	–	3	3	–
Borsod	6	9	–	4	6	–	1	5	–	–	–	–	3	3	3(2I)	1	2	2(2I)
Csongrád	1	12	3(1)	6	44	16(1)	1	1	–	–	–	–	–	–	–	2	25	–
Fejér	1	1	–	–	–	–	–	–	–	3	34	–	–	–	–	–	–	–
Győr-Sopron	–	–	–	–	–	–	1	1	–	1	1	–	–	–	–	1	1	–
Hajdú-Bihar	8	22	–	8	15	–	8	17	–	5	27	1(1I)	2	2	–	–	–	–
Heves	1	2	–	–	–	–	6	9	–	3	3	–	–	–	–	3	3	–
Komárom	2	9	–	1	48	14(1)	1	38	12(1)	2	2	1(1I)	2	5	3(1I)	4	4	–
Nógrád	4	86	14(1)	2	13	–	–	–	–	–	–	–	1	3	–	2	2	–
Pest	2	8	–	1	1	–	1	1	–	2	3	–	3	5	–	1	1	–
Somogy	7	31	2(1I)	2	19	–	10	79	9(3)	6	91	32(2)	12	129	26(1)	6	126	–
Szabolcs	3	17	4(2I)	3	5	1(1I)	1	1	–	–	–	–	2	25	12(1)	3	33	–
Szolnok	5	10	–	2	10	–	2	39	–	–	–	–	1	1	–	1	2	–
Tolna	1	3	–	–	–	–	–	–	–	–	–	–	–	–	–	1	6	–
Vas	–	–	–	1	3	–	2	5	–	–	–	–	–	–	–	–	–	–
Veszprém	2	19	–	1	1	–	2	7	–	–	–	–	–	–	–	–	–	–
Zala	7	17	–	3	11	–	2	7	–	2	7	–	15	45	–	6	15	–
Total	69	402	24	51	215	32	50	283	21	44	267	50	62	349	45	47	335	19

*Total number of strains isolated (in parentheses : I = number of strains isolated from individual household stock; numbers not marked with I refer to strains isolated from large farms)

In the period between 1988 and 1993, paraallergic reactions occurred in 323 communities, farms or household stocks (Table 1). A total of 1,848 animals from these places were slaughtered for diagnosis, and their samples were tested by laboratory methods. Samples of 191 cattle from 25 foci of infection yielded 191 mycobacterium strains (Table 2). On a glycerol-free medium incubated at 37 °C, these strains produced non-pigmented, eugonic colonies in 39–42 days, and showed acid- and alcohol-fast staining. The strains did not split NO_3, were negative in the niacin test and resistant to 2-thiophenecarboxylic hydrazide (TCH). Of the carbonic amides, the strains could utilize urea while they could not split the other substrates.

Table 2

Occurrence of bovine tuberculosis on farms of different type

Year	Household stock	Co-operative farms	State farms
1988	Szentdénes	Makó	
	Szulok	Bercel	
	Nyíregyháza		
	Kálmánháza		
1989	Kétsoprony	Makó	Szomor
	Győrtelek		
1990		Barcs	Szomor
1991	Szentdénes	Homokszentgyörgy	Mezőhegyes
	Debrecen		Bárdudvarnok
	Héreg		
1992	Somogyviszló	Csengerújfalu	Bárdudvarnok
	Szirmabesnyő	Homokszentgyörgy	
	Komlóska		
	Héreg		
1993	Edelény	Mozsgó	
	Edelény	Kétújfalu	

Subcultures of strains from the 25 foci of infection gave a positive reaction in the DNA-DNA hybridization test. By biochemical tests, the isolates were identified as *M. bovis*.

On several cattle farms, infection could not be eradicated in the year of detection. This is why the same focus of infection was found in several consecutive years (e.g. Makó, Szomor, Homokszentgyörgy, Bárdudvarnok). As regards the household farms, tuberculotic infection occurred in several household stocks of the same community (Szentdénes, Héreg, Edelény) within a year and also in different years.

Table 3

Tests for mycobacteria in different cattle herds of Baranya County between 1988 and 1993

Locality	1988	1989	1990	1991	1992	1993	Total	*M. bovis*
SF of Szentlőrinc	38	1	5	5	12	8	69	
SF of Szigetvár		11	53		4	8	76	
SF of Sóskút					2		2	
AC of Görcsöny	8						8	
AC of Vajszló	34	8	2				44	
AC of Felsőszentmárton	2	1					3	
AC of Drávafok	4	1					5	
AC of Sásd	8			4			12	
AC of Bicsérd		2		4		6	12	
AC of Újpetre		1		4			5	
AC of Szentlőrinc			3		16	2	21	
AC of Selye				4			4	
AC of Csányoszró				2			2	
AC of Kétújfalu				3		31	34	6
AC of Pécs				4			4	
AC of Rózsafa				2	23	28	53	
AC of Vejti					29		29	
AC of Mozsgó					2	15	17	11
AC of Szigetvár					9		9	
AC of Kátoly					4	8	12	
AC of Újmohács						2	2	
Household stock	4	5	4	2	6	4	25	
Total	98	30	67	34	107	112	448	17

SF = State Farm; AC = Agricultural Co-operative

Of the data of Table 1, only those regarding the tuberculin tests performed in Baranya County between 1988 and 1993 are shown in detail in Table 3. During the six years of the study involving cattle from 3 state farms, 18 agricultural co-operatives of villages and 25 household stocks of different villages, *M. bovis* infection was demonstrated only in 2 cases (Kétújfalu and Mozsgó). In all other cases, attempts to isolate *M. bovis* from organ samples taken from tuberculin-positive animals failed. Positive allergic reactions gradually disappeared also from the farms involved; however, to confirm their freedom from tuberculosis and to

determine the origin of positive tuberculin reactions, the farmers had 400 breeding animals slaughtered.

Discussion

Eight to 14 years after the completion of tuberculosis eradication (i.e. after 3–5 generations of cows of average lifespan), veterinarians evaluating positive reactions in the tuberculin tests must consider several factors including the geographical conditions, the *M. terrae, M. gordonae, M. fortuitum, nocardia* and *shagnum* infection of the soil, pasture and water, the mycobacterial infections of farmed and game animals (mammals and birds), the conditions of animal husbandry, the use of management systems without litter, with deep litter and loose housing conditions, as all these factors may facilitate the development of parallergy. The technical inaccuracy of tuberculin tests carried out in pens, which may lead to false negative results, must also be taken into consideration.

The development of parallergy is markedly influenced by the personal conditions. The intellectual, professional and hygienic demands and standard of persons employed by the farms are usually not in proportion to the value of the biological asset entrusted to them. With the change in ownership conditions and the instability of the economic regulations the protection of tuberculosis-free cattle herds has weakened. Occasionally, humans excreting different mycobacteria (persons taking immunosuppressive or antiallergic drugs or, possibly, HIV-infected individuals) may come into contact with the herds.

In the cases studied by us, *M. bovis* infections were caused by TCH-resistant strains. When tracing the source of infection by the TCH-resistance tests described by Iwainsky and Käppler (1974), it was found that the infections had mostly been caused by persons working on the reconstruction of farms or by workers employed without previous health checks. A TB-rehabilitated animal attendant, a cowman affected by lung carcinoma and simultaneously infected by *M. bovis,* a feed transport worker suffering from renal tuberculosis, an asthmatic night-watchman and an occasional hand of deviant personality also acted as sources of infection. When investigating the origin of *M. bovis* infections, in no case could we trace the infection back to animals, either to those of the same or of a different species.

Until the origin of positive tuberculin tests is elucidated, but at least until *M. bovis* infection is ruled out, the veterinary authorities must impose restrictions on the movement of animals and persons, on the sale of animals and milk, and must order isolation, diagnostic slaughter, isolated slaughtering, and repeated tuberculin testing in order to prevent additional losses and to enforce the public health requirements.

The data presented above demonstrate that the difficulties and costs of maintaining tuberculosis-free status can be determined. The smaller part of these

costs should be met by the state budget while the larger part by the farmers themselves.

Rapid and accurate diagnosis may greatly facilitate the reduction of losses arising from *M. bovis* infections.

Besides rendering the results of gel diffusion tests and bacteriological examinations more accurate, the *M. bovis* and *M. paratuberculosis* DNA-DNA hybridization test, amplification test, tumour necrosis factor and gamma interferon assays have also shortened the time of testing, thus contributing to the reduction of losses. The shortened testing time has reduced the losses arising from the restrictive measures, decreased the condemnation rate during meat inspection and, in some cases of *M. bovis* infection, it even allowed the use of tuberculosis eradication by selection rather than by herd replacement.

Acknowledgements

Specialists from the Department of Bacteriology of the National Institute for Tuberculosis and Pulmonology, the late Dr. István Szabó, Doctor of Medical Sciences, Dr. Edit Vandra, PhD, Dr. Tamás Fodor, PhD, and their colleagues have rendered an invaluable help regularly, willingly and with great professional thoroughness in the epidemiological evaluation of the situations surveyed in this work and in tracing the sources of infection. Grateful thanks are due to them for their co-operation.

I would also like to thank my fellow veterinarians supervising cattle herds and meat inspections. It is their indefatigable and accurate work which ensures the maintenance of the qualification which reads: "the cattle population of Hungary is free from tuberculosis".

References

Collection of New Animal Health and Food Hygienic Regulations (1982): Újabb Állategészségügyi és Élelmiszerhigiéniai Jogszabályok Gyűjteménye (in Hungarian). Hungexpo, Budapest, pp. 157–167.

Dénes, L. (1983*a*): Current status of bovine tuberculosis in Hungary I. Losses caused by bovine tuberculosis (in Hungarian, with English abstract). Magyar Állatorvosok Lapja **38,** 11–13.

Dénes, L. (1983*b*): Current status of bovine tuberculosis in Hungary III. The practice of eradication of bovine tuberculosis (in Hungarian, with English abstract). Magyar Állatorvosok Lapja **38,** 131–135.

Dénes, L. (1983*c*): Current status of bovine tuberculosis in Hungary IV. Statistics, conclusions, lessons learned (in Hungarian, with English abstract). Magyar Állatorvosok Lapja **38,** 195–201.

Epignost GmbH: Amplified TB-test. Gen Probe. Epignost GmbH, A-4060 Linz-Leonding, Austria.

Iwainsky, H. and Käppler, W. (1974): Mycobacterien. Johan Ambrosius Barth, Leipzig. 160 pp.

Körmendy, B. (1994): The effect of vaccination on the prevalence of paratuberculosis in large dairy herds. Vet. Microbiol. **41,** 117–125.

Körmendy, B., Nagy, Gy. and Illés, J. (1984): Cross-reactions between *Mycobacterium avium* and *Mycobacterium paratuberculosis* strains in complement fixation and gel precipitation tests. Acta Vet. Hung. **32,** 3–7.

Lepper, A. and Corner, L. (1983): Types of relationship between mycobacteria and animals. In: Ratledge, C. and Stanford, J. (eds) The Biology of the Mycobacteria. Vol. 2. Academic Press, London. pp. 420–421.

Tsukamura, M. (1975): Identification of Mycobacteria. Res. Lab. of the Nat. San. Chubu, Obu, Aichi-Ken, Japan, p. 474.

Acta Veterinaria Hungarica 43 (4), pp. 385–392 (1995)

THE VALUE OF IMMUNODIAGNOSTIC TESTS IN DETECTING TUBERCULOSIS IN AN INFECTED RED DEER HERD AND IN ERADICATION OF THE DISEASE BY SELECTION

Z. ZOMBORSZKY[1], B. KÖRMENDY[2], S.TUBOLY[3], P.TILLY[3] and P. HORN[1]

[1]Faculty of Animal Science, Pannon University of Agricultural Sciences, H–7401 Kaposvár, P.O. Box 16, Hungary; [2]Central Veterinary Institute, Budapest, Hungary; [3]Department of Epizootiology, University of Veterinary Science, Budapest, Hungary

(Received March 9, 1995)

The sensitivity and specificity of intradermal tuberculin test (Tb test) were studied in an infected red deer (*Cervus elaphus*) herd (n = 109) in comparison to the results of other examinations including gross pathology, histopathology, *Mycobacterium bovis* culture, and the lymphocyte-stimulation test (LST). In our case, relative sensitivity was 79.5% and relative specificity was 45.7%. The predictive value in negative and positive cases was 80.0% and 44.9%, respectively. On the basis of the results obtained, eradication of tuberculosis by the selection procedure from a herd of newly captured and colonized deer was performed using the Tb test. For the purposes of eradication by selection all deer responding to B-PPD were considered positive.

Key words: *Cervus elaphus*, immunodiagnostic tests, red deer, tuberculosis

Little is known about tuberculosis of Hungarian game populations, in respect of both its species and regional distribution and epizootiological role.

Occurrence of cervine tuberculosis has been reported from all over the world among others in fallow deer (*Dama dama*) kept in a deer park (Quinn and Towar, 1963), axis deer (*Axis axis*) maintained in a zoo (Jones et al., 1976), feral red deer (*Cervus elaphus*) (Dodd, 1984, Livingstone, 1980) and farmed deer including red deer (De Lisle and Havill, 1985), fallow deer (Robinson et al., 1989), sika deer (*Cervus nippon*) (Shilang and Shanzhi, 1985), and elk (*Cervus elaphus canadiensis*) (Fanning and Edwards, 1991).

Experience gained during an eradication program on a red deer farm in connection with the diagnosis of red deer tuberculosis is summarized in this paper.

0236–6290/95/$ 5.00 © 1995 *Akadémiai Kiadó, Budapest*

Materials and methods

Animals

First herd. The value of intradermal tuberculin test (Tb test) was studied on a total of 109 captured red deer (34 calves, 49 hinds and 26 stags) that had been kept and bred on a farm for two years.

Second herd. Eradication by selection was carried out among calves (n = 42) derived from the first hind population, among red deer captured and introduced to the farm in the meantime (n = 87), and among their offspring.

Tuberculin (Tb) test

In view of the stress susceptibility and seasonal reproductive characteristics of the species, the intradermal tuberculin test was carried out after the roaring, rutting and calving season, allowing for a 120-day period of desensitization.

The deer were immobilized for the test in a crush. To enable Tb testing, the hair on the side of the neck was cut with electric scissors on an area of 8 × 8 cm, separately for each antigen. The dose of antigen was 0.1 ml with both the 1 mg/ml bovine purified protein derivative (B-PPD) and the 0.5 mg/ml avian PPD (A-PPD). The test was read in the 72nd hour.

Postmortem examination

Pathological lesions typical of tuberculosis were looked for in the lymph nodes considered to be predilection sites in deer of the infected herd (n = 109) and those that reacted to B-PPD during eradication by selection (n = 36).

Histopathological examination

Samples for histopathological examination were fixed in PBS solution containing 10% formaldehyde. Lymph node and lung samples were examined after staining with haemalum and by Ziehl-Neelsen stain.

Bacteriological examination

Isolation of mycobacteria was attempted after decontamination of the samples with Sputofluol (Merck), on glycerol-containing and glycerol-free Petragnani medium. The media were kept at 37 °C for 90 days and colony development was evaluated every 10 days. Colonies that grew out and subcultures made from them were stained by the Ziehl-Neelsen procedure and identified on the basis of the time of development, pigment production, NO_3 reduction, niacin and TCH resistance test, and the carbonamido utilization test recommended by Bönicke.

Lymphocyte stimulation test (LST)

From heparinized blood samples the lymphocytes were separated by gradient centrifugation with Ficoll-Paque, suspended in Hanks' solution containing 10% fetal calf serum to give a cell density of 10^6/ml, and 1.8 ml aliquots of the suspension were measured into two Leighton tubes. To one of the tubes 200 μg/ml B-PPD was added, while to the other 0.2 ml PBS was measured (control). The rate of blastogenesis was determined on 100 counted cells and expressed in per cent.

Results

First herd

Sixty-nine of the 109 red deer subjected to comparative Tb testing reacted to B-PPD (Table 1). Of them, 41 animals had a skin swelling ranging between 3.0 and 8.9 mm ($\bar{x} \pm$ S.D. = 4.9 ± 1.6).

At postmortem examination, abscesses suspicious of tuberculosis were observed in 22 cases. Three of these cases were generalized forms, while 18 were restricted to the mesenteric lymph nodes and only 1 to the lungs and peribronchial lymph nodes. Six out of the 22 animals failed to respond to B-PPD. Two of these six deer were considered anergic also clinically on the basis of their body condition and abscess formation in the subcutaneous connective tissue in different regions of the body.

Histopathological examination revealed the presence of Langhans' giant cells of pathognostic value in organ samples from 28 animals, 8 of which had not responded to B-PPD.

Of the 22 deer with abscess formation, 13 were found to have Langhans' giant cells of pathognostic value, 4 showed epithelioid cellular changes suggestive of tuberculosis, while the remaining 5 were histopathologically negative.

Acid fast organisms were demonstrable by Ziehl-Neelsen stain in four cases in smears only.

Mycobacterium culture. *Mycobacterium bovis* was identified in 34 cases. In non-reactor animals, the histopathological demonstration of Langhans' giant cells of pathognostic value showed good agreement with the isolation of *M. bovis*. In the reactors, however, a difference was found between the results of the two methods, and the ratio of agreement was only 57.7% (15/26).

On the basis of the pathognostic changes demonstrated, in the case presented here the sensitivity of B-PPD was 79.5% (31/39) while its specificity was 45.7% (32/70). B-PPD had a predictive value in negative cases of 80.0% (32/40), and a predictive value in positive cases of 44.9% (31/69).

In the experiment aimed at determining the reliability of the lymphocyte-stimulation test, 12 B-PPD reactors had a $\geq 5\%$ index of blastogenic transforma-

tion (6.75 ± 2.08, range 5–11%). In two deer with generalized tuberculosis the rate of blastogenic transformation was 10 and 11%, respectively.

Second herd

During eradication by selection, deer responding to B-PPD with a visible or palpable skin swelling were slaughtered (Table 2).

At the first testing, one of the calves and 6 of the captured deer responded. The latter had an LST index ranging between 5 and 14% ($\bar{x}$ ± S.D. = 9.5 ± 7.25). On the basis of the pathognostic changes in this case the positive predictive value of Tb test reactor animals was 42.9% (3/7).

At the second testing, a total of 23 deer (9 of the 10-month-old growing animals and 14 captured deer) were found to respond to B-PPD. Eleven deer responded with a skin swelling > 3.0 mm, ranging between 3.3 and 18.3 mm ($\bar{x}$ ± S.D. = 5.7 ± 4.08). One of the two deer exhibiting abscess formation responded to B-PPD with a skin swelling of 5.0 mm while the other with that of 18.3 mm. In the former animal the lesions were restricted to the lungs and the peribronchial as well as mediastinal lymph nodes, while in the latter to the retropharyngeal lymph nodes. Histopathology and *M. bovis* culture were positive for both deer.

From organ samples of further 4 animals one *M. avium*, two M. *avium-intracellulare* and one *M. gordonae* strains were isolated. The reaction to B-PPD was ≤ 2.0 mm in these four deer. On comparative testing, A-PPD failed to indicate sensitized state in two cases. Seventeen animals proved negative by other examinations. At the same time, 17 out of the 18 deer (94.4%) subjected to LST had a ≥ 5% LST index (9.0 ± 11.28, range :5–16%).

At the third testing exclusively B-PPD was used as antigen (single intradermal skin test, ST). No reactor with a skin swelling > 3.0 mm was found among the 5 reactor animals. LST index ≥ 5% ($\bar{x}$ ± S.D. 80.25 ± 1.92, range 5–10%) was found in 80% of the reactors (4/5). At the same time, 15 out of the 101 non-reacting adult deer (14.85%) had an LST index ≥ 5% ($\bar{x}$ ± S.D. 5.6 ± 0.37, range 5–7%).

At the fourth testing, a reaction < 2.0 mm was palpated in 1 out of the 115 deer tested. The LST index of that animal was < 5%.

At postmortem examination of the 6 deer slaughtered on the basis of the 3rd and 4th Tb test no lesions were found. Their histopathological and bacteriological examination also proved negative.

Table 1

Summary of the diagnostic results in an infected herd of 109 red deer (first herd)

Tb test B-PPD 0.1 ml of 1 mg/ml skin reactions at 72 hours	No. of deer	Other examinations								Pathognostic changes			Negative
		Gross pathology			Histopathology			*M. bovis* culture (*M.b.*)		L+ *M.b.*+	L– *M.b.*+	L+ *M.b.*–	L– *M.b.*–
		No visible lesion	Swollen lymph nodes	Abscess-type lesion	Negative	Epithelioid cells	Langhans' giant cells (L)	Negative	Positive				
Non-reactors	40	28	6	6	23	9	8	32	8	8	–	–	32
Reactors:													
< 2.0 mm	17	10	5	2	9	3	5	12	5	4	1	1	11
2.0–3.0 mm	11	10	–	1	7	1	3	8	3	2	1	1	7
> 3.0 mm	41	12	16	13	22	7	12	23	18	9	9	3	20
($\bar{x}$±S.D. 4.9±1.6)													
All reactors	69	32	21	16	38	11	20	43	26		31		38
Total	109	60	27	22	61	20	28	75	34		39		70

Table 2

Experience of eradication by selection in captured red deer and calves of infected hinds (second herd)

Series of Tb test (method)	All deer	No. of reactors		LST ≥ 5%	Pathognostic changes	
		B-PPD	A-PPD		Langhans' cells	*M. bovis* culture
I (comparative)	97	7	5	100% (6/6)		
< 2.0 mm		4	4		2	–
2.0 – 3.0 mm		2	1		1	1
> 3.0 mm		1	–		–	–
II (comparative)	90	23	9	94.5% (17/18)		
< 2.0 mm		8	2		–	–
2.0 – 3.0 mm		4	5		–	–
> 3.0 mm		11	2		2	2
III (single)	138	5		80.0% (4/5)		
< 2.0 mm		1			–	–
2.0 – 3.0 mm		4			–	–
> 3.0 mm		–				

Discussion

The rearing of red deer in confinement has called for methods of controlling its different infectious diseases and for procedures enabling their reliable diagnosis also in Hungary. Captured hinds are more susceptible to emotional stimuli and stressors and, thus, have four times higher serum cortisol concentrations than red deer hinds reared by artifical technology (Zomborszky et al., 1993). During investigations into the tuberculosis of free-living red deer, *M.bovis* infection was diagnosed in 2 out of 21 deer by Tb testing carried out on the night of capture (Zomborszky et al., 1992). Thus, besides humans, cattle, wild boars and badgers, red deer is also a potential source of infection. Infection may show extremely rapid spread under the new management conditions differing from the natural requirements of the species and, because of the possibly large number of pre- and anergic animals, this may add to the diagnostic difficulties. Using 0.1 ml 1 mg/ml bovine PPD (Weybridge, U.K.), a relative sensitivity of 80.0% and a relative specificity of 61.3% was obtained. The test had a predictive value in negative cases of 82.6%, and a predictive value in positive cases of 57.0% (Stuart et al., 1988). In our case, relative sensitivity was 79.5% and relative specificity was 45.7%. The predictive value in negative and positive cases was 80.0% and 44.9%, respectively.

In harmony with what was reported by other authors (Beatson, 1985, Fleetwood et al., 1988), in the most severe cases the postmortem examinations revealed abscesses which in our case were restricted first of all to the mesenteric lymph nodes.

The sensitivity of the lymphocyte transformation (LT) assay was reported to be 94% while its specificity ranged between 78 and 99% depending on herd infection status (Griffin et al., 1991). In this study, 80–100% of the deer responding to B-PPD showed a $\geq$ 5% rate of blastogenic transformation as compared to the control samples.

For the purposes of eradication by selection, all deer responding to B-PPD were considered positive. At the first and second Tb testing the ratio of pathognostic changes was 3.0% and 2.2%, respectively. The ratio of deer responding to the third and fourth Tb test was below 4% and 1%, respectively, and none of the deer had a skin swelling exceeding the positivity limit applied to cattle ($\geq$ 3.0 mm). By other examinations pathognostic changes were not demonstrable in any of the cases.

From the results it appears that by the systematic use of immunodiagnostic procedures red deer, a species new for animal breeding, can be successfully rendered free from tuberculosis by the selection method, provided that the ratio of pathognostic changes does not exceed 3%. A regular and constant control of farmed red deer populations by Tb testing seems to be essential for maintaining Tb free status.

References

Beatson, N. S. (1985): Tuberculosis in red deer in New Zealand. Biology of Deer Production. The Royal Society of New Zealand, Bulletin **22,** 147–150.

De Lisle, G. W. and Havill, P. F. (1985): Mycobacteria isolated from deer in New Zealand from 1970–1983. New Zealand Vet. J. **33,** 138–140.

Dodd, K. (1984): Tuberculosis in free-living deer. Vet. Rec. **115,** 592–593.

Fanning, A. and Edwards, S. (1991): TB and deer farming: return of the King's Evil? The Lancet **338,** 1243–1244.

Fleetwood, A. J., Stuart, F. A., Bode, R. and Sutton, J. P. (1988): Tuberculosis in deer. Vet. Rec. **123,** 279–280.

Griffin, J. F. T., Cross, J. P. and Buchan, G. S. (1991): Laboratory assay for the diagnosis of tuberculosis in farmed red deer (*Cervus elaphus*). In: Brown, R. D. (ed.) The Biology of Deer. Springer-Verlag, Berlin. pp. 130–136.

Jones, D. M., Manton, V. J. A. and Cavanagh, P. (1976): Tuberculosis in a herd of axis deer (*Axis axis*) at Whipsnade Park. Vet. Rec. **98,** 525–526.

Livingstone, P. G. (1980): The evaluation of tuberculin tests in a tuberculous farmed red deer *Cervus elaphus* herd in New Zealand. Thesis for Master of Preventative Veterinary Medicine. University of California, Davis.

Quinn, J. F. and Towar, D. (1963): Tuberculosis problem in a deer park in Michigan. Am. Vet. Med. Assoc. Sci. Proc. **100,** 262–264.

Robinson, R. C., Phillips, P. H., Stevens, G. and Storm, P. A. (1989): An outbreak of *Mycobacterium bovis* infection in fallow deer (*Dama dama*). Aust. Vet. J. **66,** 195–197.

Shilang, Z. and Shanzhi, W. (1985): Prevention of tuberculosis in sika deer (*Cervus nippon*). Biology of Deer Production. The Royal Society of New Zealand Bulletin **22,** 154.

Stuart, F. A., Manser, P. A. and McIntosh, F. G. (1988): Tuberculosis in imported red deer (*Cervus elaphus*). Vet. Rec. **122,** 508–511.

Zomborszky, Z., Hafner, J. and Fehér, T. (1993): Values of serum-cortisol concentration — studies on the stress-sensibility and adaptation ability of red deer with intensive keeping (in Hungarian, with English abstract). Magyar Állatorvosok Lapja **47,** 91–96.

Zomborszky, Z., Körmendy, B., Sugár, L., Tilly, P. and Tuboly, S. (1992): Tuberculosis in captive red deer. Verh. ber. Erkrg. Zootiere. **34,** 43–46.

Acta Veterinaria Hungarica 43 (4), pp. 393–400 (1995)

DETECTION OF OCHRATOXIN A IN HUMAN BLOOD AND COLOSTRUM*

F. KOVÁCS[1], Gabriella SÁNDOR[2], A. VÁNYI[3+], S. DOMÁNY[4] and Melinda ZOMBORSZKY-KOVÁCS[5]

[1]Hungarian Academy of Sciences, H–1051 Budapest, Nádor u. 7; [2]Department of Pharmacology and [3]Department of Animal Hygiene, University of Veterinary Science, Budapest; [4]"Mór Kaposi" Hospital of Somogy County, Kaposvár; [5]Faculty of Animal Science, Pannon University of Agricultural Sciences, Kaposvár, Hungary

(Received October 11, 1994)

Ochratoxin A (OA) is one of the most frequent sources of mycotoxin contamination of feed plants in Hungary. It is produced by 10% of *Aspergillus* and 12% of *Penicillium* species, i.e. by the widely occurring "commonest" mould species. Human exposure to mycotoxins closely resembles that of swine. Fifty-two out of 100 human blood samples collected at random (52%) were found to contain ochratoxin A (0.2–12.9 ng/ml). Recent studies have clearly shown that OA has mutagenic, teratogenic and carcinogenic effects. Sensitivity to mycotoxins is known to be inversely related to age. Therefore, it was considered important to test human colostrum samples for OA content. Thirty-eight out of 92 colostrum samples (41.3%) collected from women in the first 24 hours post partum contained ochratoxin A (0.2–7.3 ng/ml). The HPLC method applied in this study is described in detail.

Key words: Ochratoxin A, human plasma, human colostrum, mycotoxin, analysis

Ochratoxin A (OA) is one of the commonest naturally occurring mycotoxins. It can be synthesized by numerous species of the genera *Aspergillus* and *Penicillium*. In studies conducted in Hungary (Sándor, 1984), 10 out of 100 *Aspergillus* strains and 12 out of 100 *Penicillium* strains isolated from feed samples were found to produce OA. Its high prevalence and incidence can be attributed partly to the rather high resistance of both *Penicillium* and *Aspergillus* strains to environmental conditions (relative humidity, temperature). This accounts for the occurrence of these moulds under both continental and tropical climatic conditions. Cereals (wheat, barley, oat, millet, maize) often serve as substrates, but

* This study was conducted in the framework of project no. 1246 of the National Research Fund (OTKA)

+ to whom reprint requests should be addressed

0236–6290/95/$ 5.00 © 1995 *Akadémiai Kiadó, Budapest*

these fungi grow well on any other type of organic matter. This means that any feed or foodstuff may be contaminated with OA.

Humans may ingest OA with foodstuffs of plant origin and secondarily also with animal products (stuffed products made from viscera, blood and plasma; eggs, etc.).

OA has nephrotoxic and hepatotoxic effects. The OA-induced nephropathy of swine was first described in Denmark (Larsen, 1928). Based upon the results of recent studies, a long-known human disease designated "Balkan endemic nephropathy" has also been assumed to be caused by OA.

Hungary produces large quantities (about 14–15 million tons) of grain crops each year. With this in view, the high prevalence of OA can easily be understood. According to chemical assays performed in the 'seventies (Sándor et al., 1982), 14–15% of feed samples submitted for testing because of some disease or production decrease were found to contain OA. This ratio of feed contamination is comparable to that demonstrated in European countries including Scandinavia and in North America (Krogh, 1980).

Because of the high prevalence of OA in Hungary, the incidence of OA-induced nephropathy was determined by a survey of almost 1.5 million swine at 4 slaughterhouses (Sándor et al., 1982). Meat inspection revealed morphological changes in 2 out of 10,000 animals, which is a ratio similar to that found in Denmark. OA could be detected chemically in 39% of the 122 kidneys showing macroscopic lesions. The degree of toxin contamination was as follows:

Table 1

Ochratoxin A levels detected in pig kidneys

OA level, μg/kg kidney	% (of positive kidneys)
< 5	66.0
5 – 10	14.9
10 – 50	8.5
50 –100	0
> 100	10.0

Pig feed and human food show a high degree of resemblance: both are based on cereals. In humans, the situation is further aggravated by the fact that they can take up OA also with foodstuffs of animal origin (Sándor et al., 1991) and that the elimination half-life of the toxin is the longest, i.e. accumulation must also be reckoned with.

These facts prompted us to collect data on the degree of human exposure to OA.

Materials and methods

Collection of samples

Blood samples were collected by a randomized method. A total of 100 samples were tested: 50 had been obtained from the patient material of the Hospital of Szolnok County (in the period from May to July) and the other 50 from the Blood Supply Unit (in the month of August). The anticoagulant-treated blood samples were centrifuged and the plasma samples thus obtained were stored at –22 °C until processed.

Mother's milk (colostrum) samples were collected at the Maternity Ward of the Hospital of Kaposvár from parturient mothers within 24 h after delivery, in the period between August and October. The samples were stored frozen at –22 °C until processed.

Determination of ochratoxin A in blood and mother's milk samples

The OA content of the blood samples was determined by the method described by Sándor et al. (1991).

The determination of ochratoxin A in mother's milk samples was carried out by the method described by Gareis et al. (1988), as follows.

Chemicals. The ochratoxin A reference material (Makor Chemicals, Jerusalem) was dissolved in methanol and adjusted to a concentration of 50 µg/ml. For the HPLC determinations, aliquots of that stock solution were taken, evaporated, then dissolved in an organic solvent of suitable volume to obtain the standard working solutions. All chemicals used were of analytical or HPLC grade.

Samples. The mother's milk samples were collected from August to October 1992. For the calibration of the method and for determining the detection limits and recovery percentages, cow's milk was used as toxin-free control.

Extraction and purification. To 2 ml milk sample, 10 ml buffer solution was added (118 g NaCl, 33.72 ml H_3PO_4 to 1,000 ml distilled water, adjusted to pH 1.6 by adding 1 M NaOH solution). After mixing, the pH of the sample was adjusted to 1.6 with 0.5 M H_3PO_4 solution. The sample was then shaken on a shaker for 1 h, then 10 ml chloroform was added and the mixture was extracted during shaking for 30 min. This was followed by centrifugation at 5,000 rpm for 10 min at 4 °C. Subsequently the mixture was allowed to separate in a separating funnel, the chloroform phase was collected in an evaporating flask, and extraction was repeated by adding another 10-ml volume of chloroform. The pooled chloroform phases were evaporated almost to dryness, the residue was suspended in 5 ml chloroform and extracted with 2 × 5 ml 1.25% $NaHCO_3$ solution. The pH of the pooled $NaHCO_3$ phase was adjusted to a value of 2 by adding concentrated formic acid. Ochratoxin A was then re-extracted with 2 × 2 ml chloroform. The solvent was evaporated in N_2 stream.

High-performance liquid chromatography (HPLC). The evaporated residue was dissolved in 250 µl methanol with the help of a test tube shaker, and 20 µl of the obtained solution was injected into the HPLC system.

The components of the HPLC system were as follow: Shimadzu LC6 pump, SCL-6A system controller, RF-540 fluorimeter with flow-through cuvette, SIL-9A autoinjector, C-R4A chromatopac integrator, µ Bondapak C18 column, 300 × 3.9 mm, 10 µm (Waters, Millipore).

Measuring conditions: Eluent: acetonitrile – distilled water – acetic acid (570+410+20). Flow rate: 1 ml/min. Excitation wavelength: 330 nm, slot: 10 nm; emission wavelength: 460 nm, slot: 20 nm. The OA content was determined with the help of a computer programme built in the integrator using the least squares method on the basis of a 4-point calibration line. Limit of detection: 0.04 ng/20 µl inj. extract. Limit of quantitation: 0.2 ng OA/ml plasma or milk.

In the expected concentration range, the recovery rates of OA were determined by analysis of a reference solution added to cow's milk. The results are shown in Table 2.

Table 2

Recovery rates of ochratoxin A added to cow's milk

Ochratoxin A, ng/ml	Recovery* %
12.50	80.6 (±13)
6.25	78.2 (± 7)
2.50	77.7 (± 4)
1.25	99.6 (±13)

*calculated from 4–6 parallel determinations

Table 3

Ochratoxin A levels detected in human blood samples

Number of samples tested	100
Of that:	
positive:	52
negative:	48
Ochratoxin A level (ng/ml):	
<1	34
1–5	16
5–10	1
>10	1
Total:	52 (52%)

Results

Blood samples. The results are summarized in Table 3. OA was detected in 52 out of the 100 blood samples assayed (52%).

Colostrum samples. Thirty-eight of the 92 colostrum samples were found to be positive. The OA content was 0.04–1.22 ng in the 20 µl volumes injected. As the extraction residue was dissolved in a volume of 250 µl, which corresponds to 2 ml milk sample, the OA content of the positive samples was between 0.22 and 7.63 ng/ml, in the following distribution:

below 1 ng/ml	13 samples
1–2 ng/ml	12 samples
2–3 ng/ml	8 samples
3–5 ng/ml	3 samples
above 5 ng	2 samples

Figure 1 shows the chromatograms of an OA reference solution (A), control cow's milk (B) and cow's milk + OA (C) after sample preparation described in the Methods.

Figure 2 shows the chromatograms of an OA reference solution (A), a positive mother's milk sample (B) and a negative mother's milk sample (C).

By comparing the chromatograms of a reference solution and a positive sample, both figures enable an easy identification of the peaks. The peaks of other "contaminating" compounds present in the purified milk extract are clearly distinguishable from the OA peak, and thus they do not interfere with the identification.

Discussion

As had been expected, OA could be detected in a relatively high proportion of both the human blood and the mother's milk samples assayed. In the Balkan countries, human exposure to ochratoxin A had been investigated already earlier; however, in view of the endemic occurrence of nephropathy in that region, the data obtained there could not be extrapolated to other areas of Europe. Breitholtz et al. (1991) analyzed human blood samples for OA in three different regions of Sweden. OA was detected in samples from all three regions; however, the degree of contamination varied widely. OA contamination is known to be high in the Scandinavian countries: this fact may account for the high degree of human exposure observed. The analytical method used by the above-cited authors also seems to have been more sensitive. In Germany, Gareis et al. (1988) detected OA in 4 out of the 36 mother's milk samples analyzed. The results obtained by us occupy an intermediate position between those of the former two teams. All in all, the results

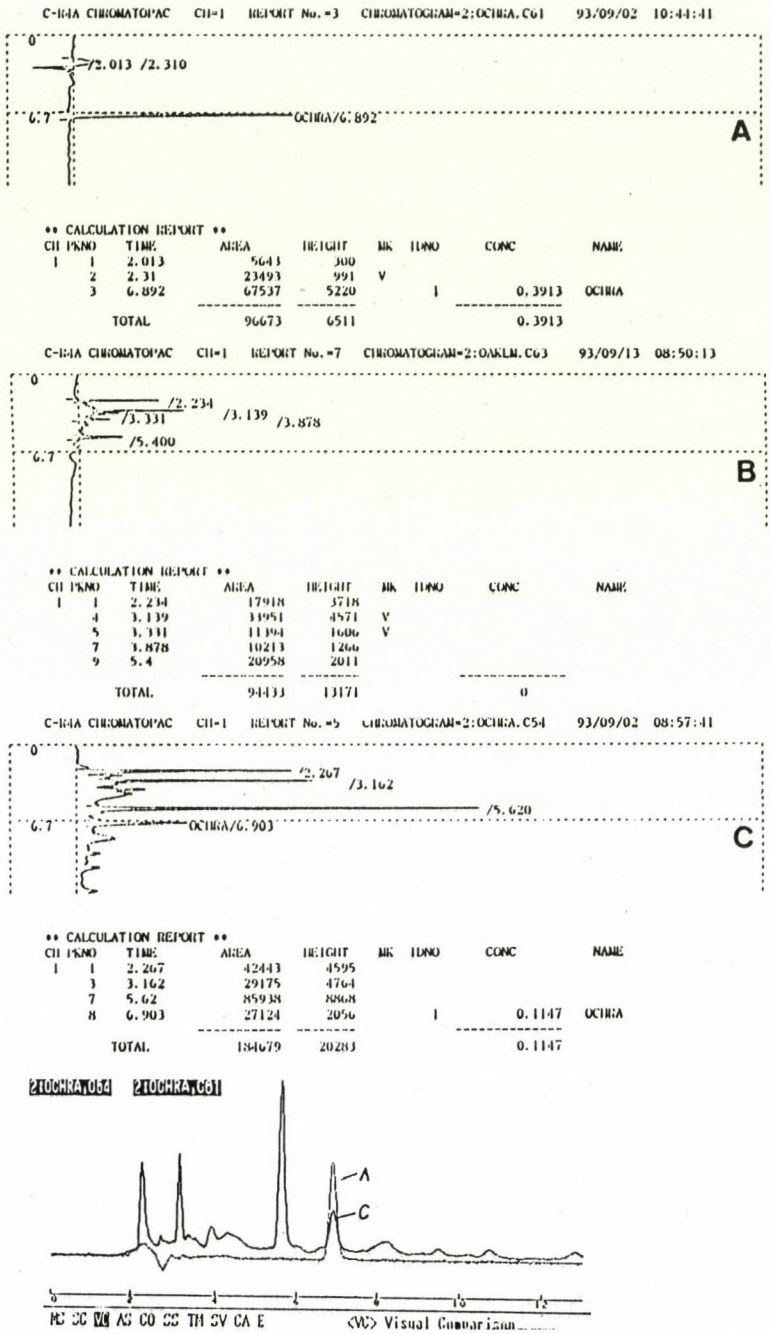


Fig. 1. Chromatograms of an ochratoxin A reference solution (A) and of extracts of control cow's milk (B) and cow's milk + ochratoxin A solution (C)

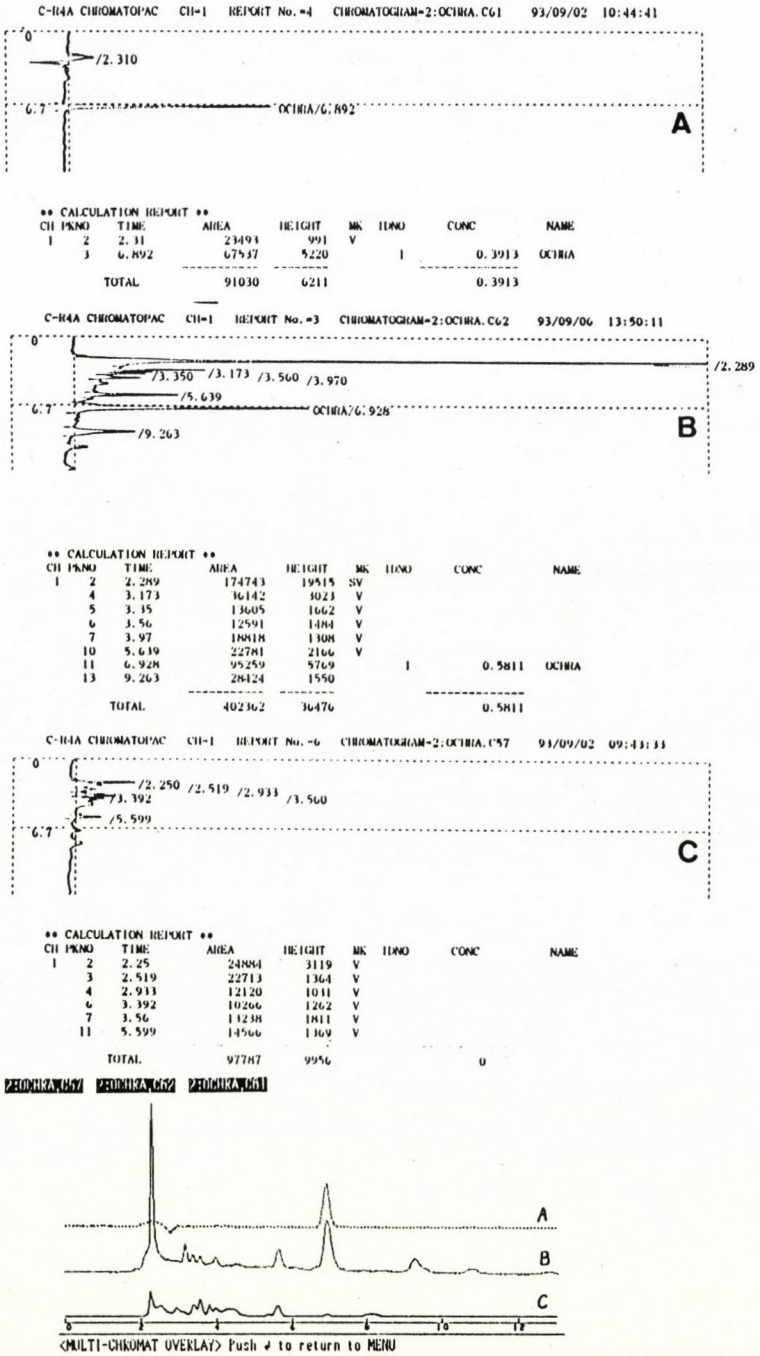


Fig. 2. Chromatograms of an ochratoxin A reference solution (A) and of extracts of positive mother's milk (B) and negative mother's milk samples (C)

indicate that human exposure to OA is a distinct possibility. This is a serious risk if we consider that now it is already a generally held view that OA has teratogenic, mutagenic and carcinogenic properties. Susceptibility to the effects of mycotoxins is inversely related to age. Mycotoxins present in the maternal organism, which are excreted also in the mother's milk in the postpartum period, pose a serious risk to the health of newborn infants.

References

Breitholtz, A., Olsen, M., Dahlbäck, A. and Hult, K. (1991): Plasma ochratoxin A levels in three Swedish populations surveyed using an ion-pair HPLC technique. Food Additives and Contaminants **8,** 183–192.

Gareis, M., Märtbauer, M., Bauer, J. and Gedek, B. (1988): Bestimmung von Ochratoxin A in Muttermilch. Z. Lebensm. Unters. Forsch. **186,** 114–117.

Krogh, P. (1980): "Natural Toxins". Pergamon Press, Oxford. pp. 673–680.

Larsen, S. (1928): Porcine nephropathy, an endemically occurring disease in Scandinavia. Manedsskr. Dyrl. **40,** 259–284.

Sándor, Gabriella (1984): Deterioration of feedstuffs by mycotoxins in Hungary. Proceedings of the 6th International Biodeterioration Symposium, Washington D.C., 1984. pp. 262–268.

Sándor, Gabriella, Busch, Angela, Watzke, H., Reek, J. and Ványi, A. (1991): Subacute toxicity testing of ochratoxin A and citrinin in swine. Acta Vet. Hung. **39,** 149–160.

Sándor, Gabriella, Glávits, R., Vajda, L., Ványi, A. and Krogh, P. (1982): Epidemiologic study of ochratoxin-A associated porcine nephropathy in Hungary. Vth Internat. IUPAC Symposium on Mycotoxins and Phytotoxins, Vienna, 1982. pp. 349–352.

MAGYAR
TUDOMÁNYOS AKADÉMIA
KÖNYVTÁRA

Acta Veterinaria Hungarica 43 (4), pp. 401–422 (1995)

DYNAMICS OF *ANGUILLICOLA CRASSUS* (NEMATODA: DRACUNCULOIDEA) LARVAL INFECTION IN PARATENIC HOST FISHES OF LAKE BALATON, HUNGARY

Cs. SZÉKELY

Veterinary Medical Research Institute, Hungarian Academy of Sciences,
H–1143 Budapest, Hungária krt. 21, Hungary

(Received March 1, 1995)

Anguillicola crassus, a nematode parasitizing the swimbladder of the European eel (*Anguilla anguilla*) caused substantial mortality among eels in Lake Balaton in the years 1991 and 1992. Parallel to a 3-year study of the infection dynamics of eels, *Anguillicola* larval infection of paratenic host fishes was also surveyed in the lake between 1991 and 1993. During that study, a total of 1,382 specimens of 22 paratenic host fish species were processed. The results showed that anguillicolosis had become a parasitosis widespread throughout the lake, and larval infection could be detected in practically all paratenic hosts examined. The prevalence and intensity values recorded in the paratenic hosts do not completely follow the dynamic change observed during the survey of eel infection. During the study of larval infection in paratenic hosts, only inter-species differences in the prevalence and intensity of infection could be found, rather than the expected course of infection spreading from the West to the East in both space and time. Marked differences existed between paratenic host species in the degree of host reaction to the larvae.

Key words: *Anguillicola crassus,* larval infection, paratenic hosts, dynamics, Lake Balaton

Since *Anguillicola crassus* was introduced into Europe at the end of the 1980's, numerous papers have dealt with its spread on the European continent (Paggi et al., 1982: Neumann, 1985; Sarti et al., 1985; Peters and Hartmann, 1986; Hartmann, 1987; Taraschewski et al., 1987; Dupont and Petter, 1988; Belpaire et al., 1989; Koops and Hartmann, 1989; Kennedy and Fitch, 1990; Køie, 1991; Székely et al., 1991; Moravec, 1992), its development (De Charleroy et al., 1990; Haenen and van Banning, 1991; Höglund and Thomas, 1992; Thomas and Ollevier, 1992), seasonal appearance (van Willigen and Dekker, 1989), pathogenic effect on the host (Boon et al., 1989; Haenen et al., 1989; Boon et al., 1990*a, b, c*; van Banning and Haenen, 1990; Molnár et al., 1991; Möller et al., 1991; Sprengel and Lüchtenberg, 1991; Höglund et al., 1992), mechanical injury caused in the swimbladder wall (Liewes and Schaminee-Main, 1987; Kamstra, 1990), and histopathological changes induced by it (Haenen et al., 1989; van Banning and

0236–6290/95/$ 5.00 © 1995 *Akadémiai Kiadó, Budapest*

Haenen, 1990; Molnár et al., 1993). Several authors (Buchmann et al., 1991; Höglund and Pilström, 1994; Haenen, 1995) have suggested that eels develop immunological resistance against *A. crassus*. Although many of the above authors have referred to the dynamics of the parasite's occurrence, detailed data can be found only in the works of van Willigen and Dekker (1989), Thomas and Ollevier (1992), Höglund and Andersson (1993) and Molnár et al. (1994).

The paratenic hosts of *A. crassus* have been studied by Cannaerts (1989), De Charleroy et al. (1990), Haenen and van Banning (1990), Thomas and Ollevier (1992), Höglund and Thomas (1992), Székely (1994), and Pazooki and Székely (1994). Of the studies reported so far, however, only that of Thomas and Ollevier (1992) dealt with the seasonal dynamics of occurrence of larvae in paratenic hosts.

These data show that anguillicolosis is a parasitosis which has been studied in much detail. Still, the study reported here was justified by the mass mortality that *A. crassus* infection had caused among eel in Lake Balaton in 1991 and 1992: the epidemiological, ecological and environmental factors contributing to such a severe manifestation of the disease in that lake had to be explored. This was done by Molnár et al. (1994) who studied the dynamics of *A. crassus* infection in the eel population of Lake Balaton between 1991 and 1993. Parallel to that survey, the prevalence and intensity of *A. crassus* larval infection in paratenic hosts were also studied, and the results are reported in this paper.

Materials and methods

The studies were conducted throughout the period between September 1991 and November 1993. A total of 1,382 small fish belonging to 22 species (paratenic hosts) were collected, parallel to the eel catches, from three different habitats of the lake (Fig. 1): from the eutrophicated Western basin (Keszthely, Badacsony), from the less eutrophicated central basin (Udvardi, Tihany, Csopak), and from the oligotrophic Eastern region (Almádi, Kenese). All samples were taken from the littoral areas of the lake. The species distribution and the number of specimens of fish processed during the three-year period are shown in Table 1.

Some of the fish were collected with the help of the Balaton Limnological Research Institute of the Hungarian Academy of Sciences and the rest by ourselves, with the help of electrofishery equipment and a trawl-net. Although we always intended to catch fishes of the same species composition and specimen number, in the practice many times only low numbers of specimens could be collected, and the samples obtained were not always of the same species composition. The sample consisted of fish of a size suitable for eel food (max. 13 cm). Fish larger than that were thrown back into the water, with the exception of the pike, of which small specimens could not be caught; therefore, some 20 to 30 cm long pikes were also processed.

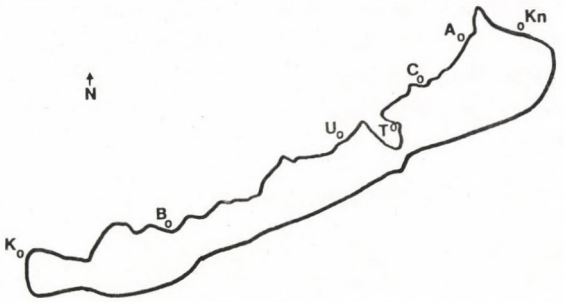


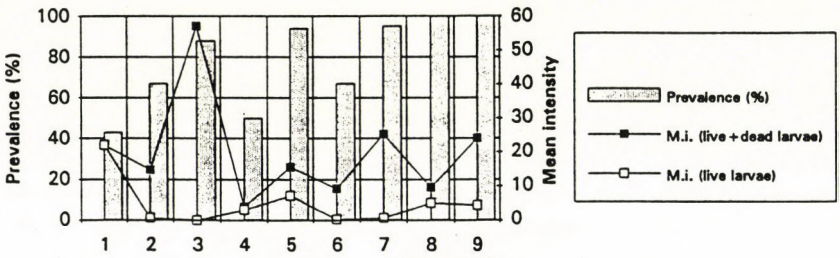
Fig. 1. Sampling places in Lake Balaton in the years 1991 through 1993 (Sampling places: Western basin: K = Keszthely, B = Badacsony; Central basin: U = Udvari, T = Tihany, C = Csopak; Eastern basin: A = Almádi, Kn = Kenese)

Table 1

Fish species examined and number of specimens processed in the period 1991–1993

Fish species		Number of specimens examined
Bleak	*Alburnus alburnus*	378
Pumpkinseed	*Lepomis gibbosus*	198
River goby	*Neogobius fluviatilis*	169
Roach	*Rutilus rutilus*	160
White bream	*Blicca bjoerkna*	78
Rudd	*Scardinius erythrophthalmus*	68
Bitterling	*Rhodeus sericeus amarus*	65
Chinese rasbora	*Pseudorasbora parva*	63
Pike perch	*Stizostedion lucioperca*	58
Bream	*Abramis brama*	38
Ruffe	*Gymnocephalus cernua*	25
Gibel carp	*Carassius auratus gibelio*	22
Gudgeon	*Gobio albipinnatus*	17
European catfish	*Silurus glanis*	14
Pike	*Esox lucius*	8
Asp	*Aspius aspius*	7
Perch	*Perca fluviatilis*	5
Common carp	*Cyprinus carpio*	3
Tench	*Tinca tinca*	3
Crucian carp	*Carassius carassius*	1
Rain bleak	*Leucaspius delineatus*	1
Brown bullhead	*Ictalurus nebulosus*	1
Total:	22 species	1,382

The fish were collected from the sampling places in the fishing period (from spring to autumn), possibly from every region in each season, and transported to the laboratory alive. The fish kept in our aquaria were processed within the shortest possible time in all cases; however, because of the time-consuming examinations the processing of a sample often took 1–2 weeks. The fish were killed, their abdominal cavity was opened, all internal organs were removed, and fresh squash preparations were made from them between two slides for light microscopic examination. Depending on the size of the internal organs, often as many as 10–15 squash preparations covering an entire slide were made. In order to recover all larvae present in the abdominal cavity the preparations were scanned by moving the microscopic field in a zigzag line. The dissection results were recorded: the records included the species and total body length of the fish and the number of live and dead larvae found in the abdominal cavity. The host reaction, if any, that developed around the larva in the paratenic host was also mentioned.

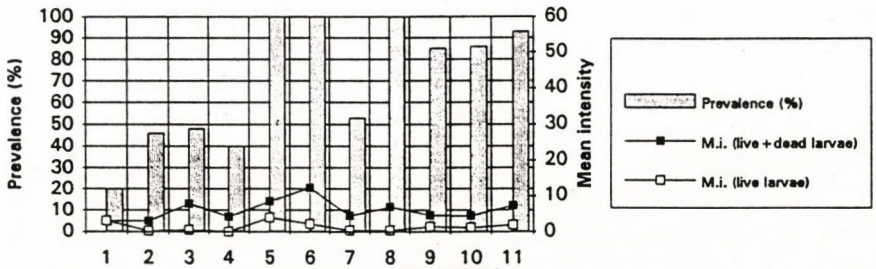


Samplings		Nr. of fish examined
1	17.09.1991.	7
2	11.03.1992.	27
3	21.04.1992.	8
4	24.06.1992.	2
5	25.08.1992.	17
6	19.04.1993.	15
7	09.06.1993	21
8	23.08.1993.	8
9	21.10.1993.	5
	Total examined	110

Chart 1.1. Prevalence and mean intensity of *Anguillicola crassus* larval infection of bleak (*Alburnus alburnus*) in the Western basin of Lake Balaton

On the basis of the records, the prevalence of infection by 3rd stage *A. crassus* larvae and the mean intensity of infection by live and dead larvae were calculated for the different fish species, and the obtained values were graphically illustrated using the Excel 4.0 programme. With the exception of one instance (Chart 10, which shows data collected in a 2-year period), the graphs (Charts 1–14) present data obtained during a 3-year period (1991–1993). In the case of some fish species of which a large number of specimens were examined (bleak, pumpkin-

seed, river goby, and roach) the dynamics of infection are illustrated separately for the three regions of the lake (Charts 1.1, 1.2, 1.3, 2.1, 2.2, 2.3, 3.1, 3.2, 3.3, 4.1, 4.2 and 4.3), whereas the data obtained for those represented by a lower number of specimens are shown in a comprehensive chart each (Charts 5–12). Data of species represented in the sample only by a few specimens were summarized in two charts (Charts 13–14).



Samplings		Nr. of fish examined
1	05.09.1991. Tihany	10
2	31.03.1992. Csopak	11
3	31.03.1992. Tihany	21
4	20.05.1992. Tihany	15
5	03.08.1992. Tihany	9
6	10.08.1992. Csopak	9
7	06.04.1993. Csopak	28
8	27.05.1993. Tihany	3
9	05.07.1993. Tihany	13
10	09.08.1993. Csopak	22
11	12.10.1993. Tihany	29
	Total examined	170

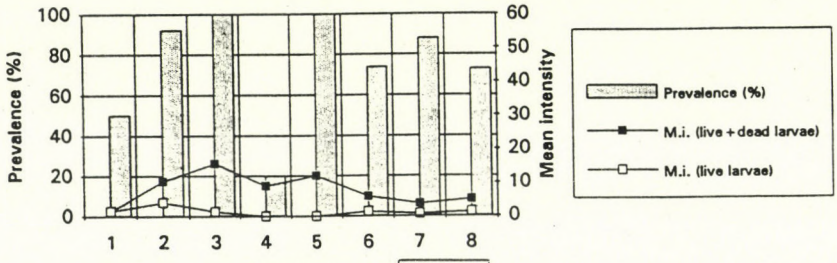
Chart 1.2. Prevalence and mean intensity of *Anguillicola crassus* larval infection of bleak (*Alburnus alburnus*) in the Central basin of Lake Balaton

Results

A surprisingly high proportion of the 1,382 fish collected from different areas of Lake Balaton and dissected in the laboratory was found to be infected by live or dead 3rd stage larvae of *A. crassus*.

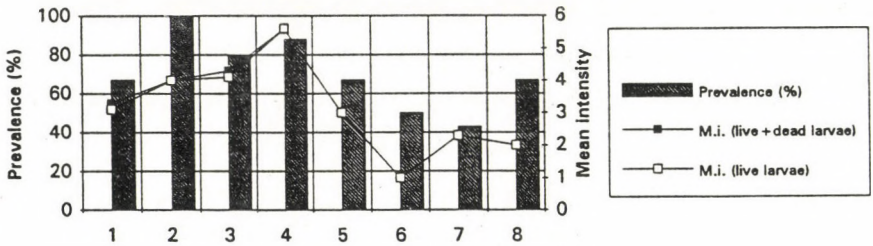
For a higher degree of transparency and comparability, the results shown in the Charts were summarized in tables rather than having been evaluated in the text. Table 2 contains the data of infection (prevalence, intensity of infection by live larvae, intensity of infection by live and dead larvae combined, expressed as ranges of the mean calculated for each sampling) of the four fish species represented in the sample by the highest number of specimens (bleak, pumpkinseed, river goby and roach), separately for the three regions of Lake Balaton. Table 3

contains similar data on paratenic host fish species caught in a lower number of specimens (white bream, rudd, bitterling, Chinese rasbora, pike perch, bream, ruffe, gibel carp, European catfish, gudgeon, pike, asp, perch, common carp, tench, crucian carp, rain bleak, brown bullhead) for the entire area of the lake.



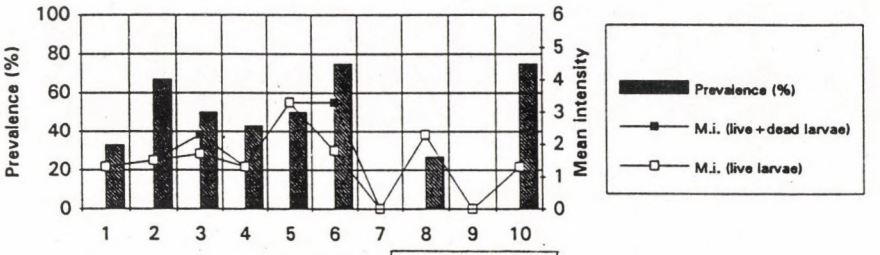
Samplings		Nr. of fish examined
1	02.10.1991. Almádi	4
2	24.07.1992. Világos	12
3	09.09.1992. Szabadi	15
4	09.09.1992. Kenese	5
5	26.04.1993. Kenese	1
6	24.06.1993. Kenese	31
7	01.09.1993. Kenese	8
8	02.11.1993. Kenese	22
	Total examined	98

Chart 1.3. Prevalence and mean intensity of *Anguillicola crassus* larval infection of bleak (*Alburnus alburnus*) in the Eastern basin of Lake Balaton



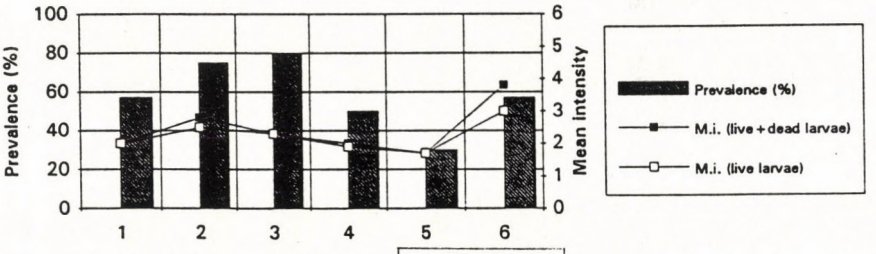
Samplings		Nr. of fish examined
1	16.09.1991.Keszthely	21
2	11.03.1992. Keszthely	1
3	21.04.1992. Keszthely	10
4	24.06.1992. Keszthely	8
5	25.08.1992. Keszthely	3
6	19.04.1993. Keszthely	2
7	09.06.1993. Keszthely	7
8	21.10.1993. Keszthely	6
	Total examined	58

Chart 2.1. Prevalence and mean intensity of *Anguillicola crassus* larval infection of pumpkinseed (*Lepomis gibbosus*) in the Western basin of Lake Balaton



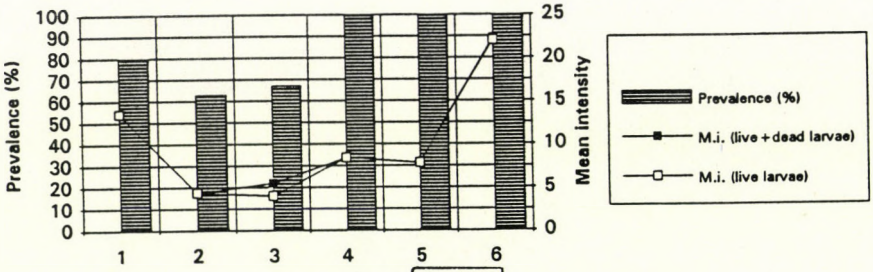
	Samplings	Nr. of fish examined
1	05.09.1991. Tihany	9
2	07.11.1991. Csopak	3
3	31.03.1992. Csopak	6
4	20.05.1992. Tihany	14
5	03.08.1992. Tihany	6
6	10.08.1992. Csopak	8
7	06.04.1993. Csopak	1
8	27.05.1993. Tihany	11
9	09.08.1993. Csopak	3
10	12.10.1993. Tihany	4
	Total examined	65

Chart 2.2 Prevalence and mean intensity of *Anguillicola crassus* larval infection of pumpkinseed (*Lepomis gibbosus*) in the central basin of Lake Balaton



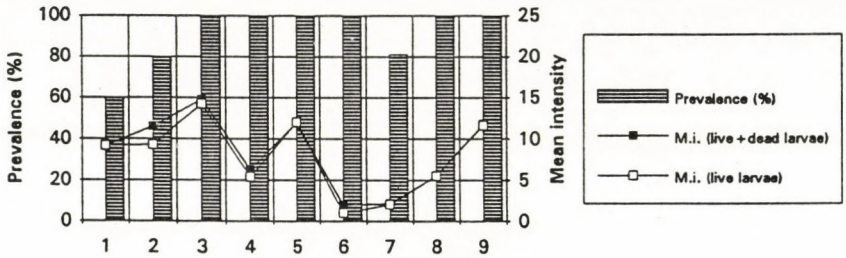
	Samplings	Nr. of fish examined
1	02.10.1991. Almádi	7
2	09.09.1992. Kenese	20
3	26.04.1993. Kenese	15
4	24.06.1993. Kenese	16
5	01.09.1993. Kenese	10
6	02.11.1993. Kenese	7
	Total examined	75

Chart 2.3. Prevalence and mean intensity of *Anguillicola crassus* larval infection of pumpkinseed (*Lepomis gibbosus*) in the Eastern basin of Lake Balaton



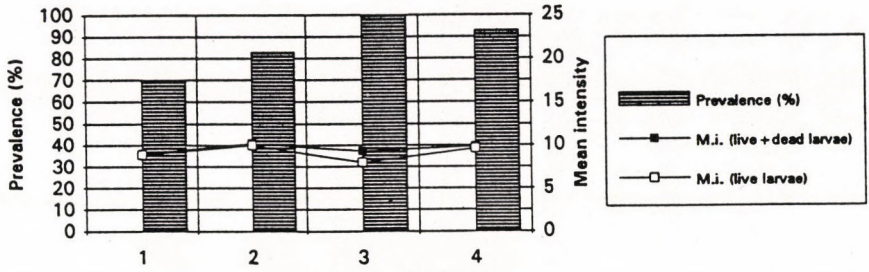
Samplings		Nr. of fish examined
1	17.09.1991.	5
2	24.10.1991.	8
3	21.04.1992.	3
4	25.08.1992.	8
5	09.06.1993.	4
6	23.08.1993.	21
	Total examined	49

Chart 3.1. Prevalence and mean intensity of *Anguillicola crassus* larval infection of river goby (*Neogobius fluviatilis*) in the Western basin of Lake Balaton



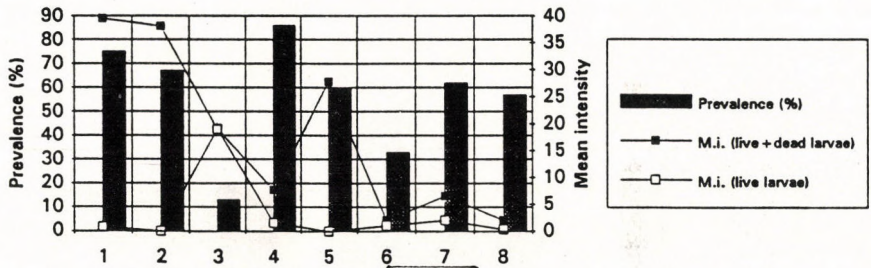
Samplings		Nr. of fish examined
1	05.09.1991. Tihany	10
2	07.11.1991. Csopak	5
3	20.05.1992. Tihany	10
4	03.08.1992. Tihany	9
5	10.08.1992. Csopak	1
6	27.05.1993. Tihany	1
7	05.07.1993. Tihany	21
8	09.08.1993. Csopak	8
9	12.10.1993. Tihany	18
	Total examined	83

Chart 3.2. Prevalence and mean intensity of *Anguillicola crassus* larval infection of river goby (*Neogobius fluviatilis*) in the central basin of Lake Balaton



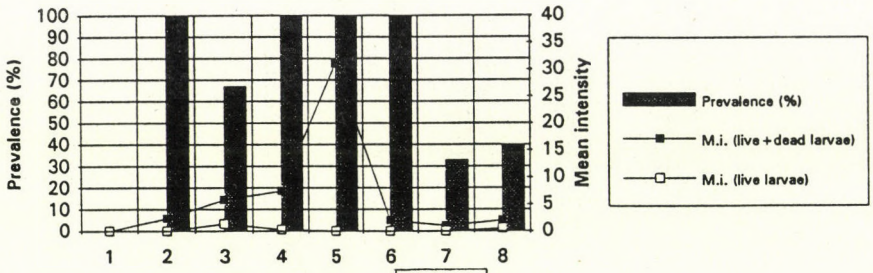
		Nr. of fish examined
1	02.10.1991. Almádi	10
2	09.09.1991. Szabadi	6
3	09.09.1992. Kenese	6
4	24.06.1993. Kenese	15
	Total examined	37

Chart 3.3. Prevalence and mean intensity of *Anguillicola crassus* larval infection of river goby (*Neogobius fluviatilis*) in the Eastern basin of Lake Balaton



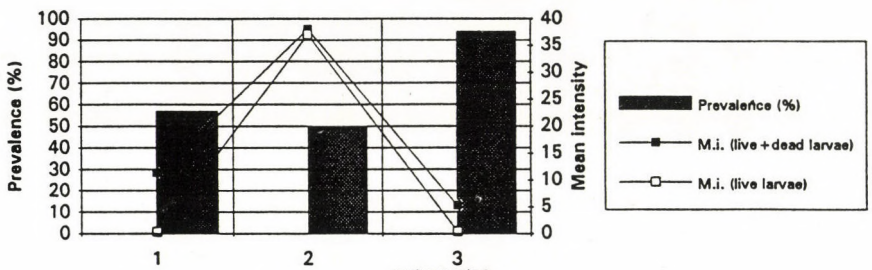
		Nr. of fish examined
1	16.09.1991. Keszthely	8
2	11.03.1992. Keszthely	15
3	24.06.1992. Keszthely	8
4	25.08.1992. Keszthely	21
5	19.04.1993. Keszthely	10
6	09.06.1993. Keszthely	3
7	23.08.1993. Keszthely	13
8	21.10.1993. Keszthely	7
	Total examined	85

Chart 4.1. Prevalence and mean intensity of *Anguillicola crassus* larval infection of roach (*Rutilus rutilus*) in the Western basin of Lake Balaton



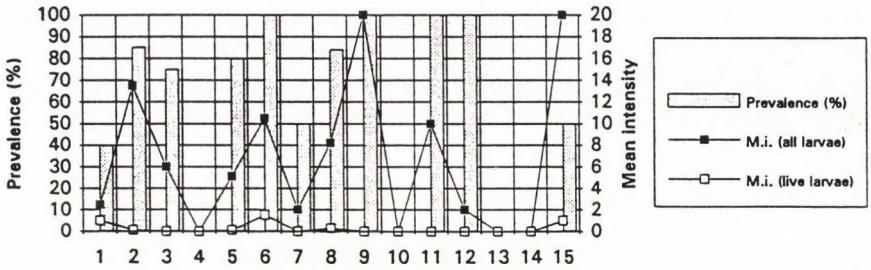
Samplings		Nr. of fish examined
1	05.09.1991. Tihany	4
2	20.05.1992. Tihany	3
3	03.08.1992. Tihany	9
4	10.08.1992. Csopak	5
5	06.04.1993. Csopak	4
6	27.05.1993. Tihany	1
7	05.07.1993. Tihany	3
8	09.08.1993. Csopak	20
	Total examined	49

Chart 4.2. Prevalence and mean intensity of *Anguillicola crassus* larval infection of roach (*Rutilus rutilus*) in the central basin of Lake Balaton



Samplings		Nr. of fish examined
1	09.09.1992. Kenese	7
2	24.06.1993. Kenese	2
3	01.09.1993. Kenese	17
	Total examined	26

Chart 4.3. Prevalence and mean intensity of *Anguillicola crassus* larval infection of roach (*Rutilus rutilus*) in the Eastern basin of Lake Balaton



Samplings		Nr. of fish examined
1	17.09.1991. Keszthely	5
2	11.03.1992. Keszthely	13
3	21.04.1992. Keszthely	4
4	24.07.1992. Világos	1
5	03.08.1992. Tihany	10
6	10.08.1992. Csopak	11
7	25.08.1992. Keszthely	4
8	09.09.1992. Kenese	19
9	06.04.1993. Csopak	1
10	26.04.1993. Kenese	1
11	24.06.1993. Kenese	1
12	05.07.1993. Tihany	1
13	09.08.1993. Csopak	1
14	23.08.1993. Keszthely	2
15	01.09.1993. Kenese	4
	Total examined	78

Chart 5. Prevalence and mean intensity of *Anguillicola crassus* larval infection of white bream (*Blicca bjoerkna*) in Lake Balaton

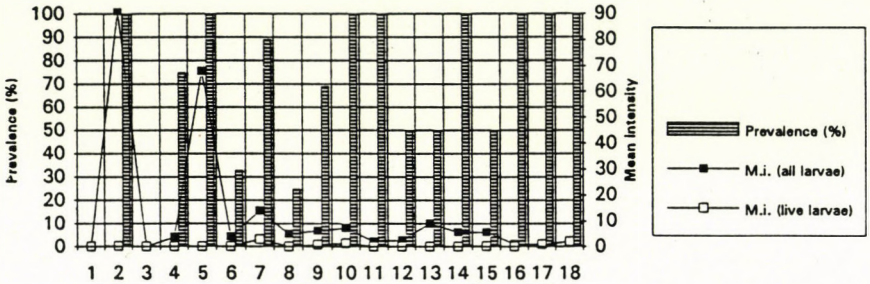
Discussion

The results presented here support the finding of Molnár et al. (1994), namely that at the time of the study anguillicolosis was a parasitosis widespread in the entire area of Lake Balaton and it caused infection not only in eels but also in practically all paratenic host fish species examined (in the form of larval infection). The prevalence and intensity of larval infection found in paratenic hosts do not completely follow the dynamic changes found by Molnár et al. (1994) during the study of eel infection. Thus, during the study of larval infection in paratenic hosts, only inter-species differences in the prevalence and primarily in the intensity of infection could be found, rather than the expected course of infection spreading in the three regions from the West towards the East in both space and time.

The biggest differences between paratenic host species occurred in the degree of host reaction to the larvae, which has already been reported by Székely (1994). As a consequence, in some species — first of all in cyprinids (Charts 1.1–1.3, 4.1–4.3, 5, 6, 7, 8 and 10) — the number of dead larvae as compared to

that of live larvae was high, while in other species — primarily in percids and in the river goby (Charts 2.1–2.3, 3.1–3.3, 9, 11) live larvae were dominant as a result of the weak host reaction. Because of the weak host reaction, in one species, the pike perch (Chart 9) no dead larvae were found.

According to the assumption of Molnár et al. (1994), the parasite first appeared in the Western part of Lake Balaton. Its rapid propagation was potentiated by the high eel density that developed as a result of the eutrophication of that region. The large number of paratenic hosts (small fishes) living in the region and in other areas of the lake also contributed to the rapid spread of infection.

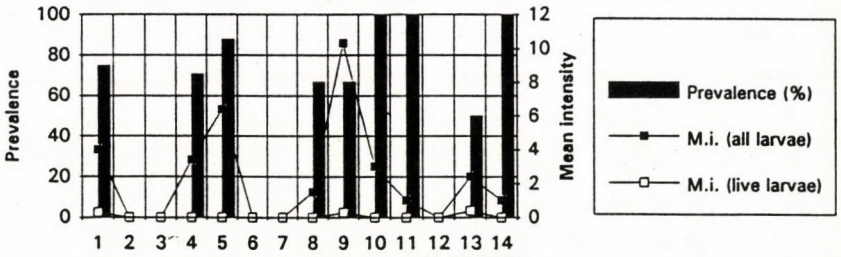


	Samplings	Nr. of fish examined
1	05.09.1991. Tihany	1
2	24.10.1991. Keszthely	3
3	02.10.1991. Almádi	1
4	11.03.1992. Keszthely	4
5	21.04.1992. Keszthely	4
6	20.05.1992. Tihany	3
7	24.06.1992. Keszthely	9
8	03.08.1992. Tihany	4
9	10.08.1992. Csopak	13
10	09.09.1992. Kenese	3
11	06.04.1993. Csopak	1
12	19.04.1993. Keszthely	4
13	09.06.1993. Keszthely	6
14	05.07.1993. Keszthely	3
15	09.08.1993. Csopak	6
16	01.09.1993. Kenese	1
17	12.10.1993. Keszthely	1
18	02.11.1993. Kenese	1
	Total examined	68

Chart 6. Prevalence and mean intensity of *Anguillicola crassus* larval infection of rudd (*Scardinius erythrophthalmus*) in Lake Balaton

Molnár et al. (1994) found that larger eels of Lake Balaton were colonized by more helminths than those of smaller size. The dissimilar number of helminths found in eels of different size can partially be explained by the fact that smaller eels consume lower numbers of infected intermediate hosts (e.g. cyclops) or parat-

enic hosts (small fish). The number of helminths colonizing eels can be markedly influenced by the degree of host reaction developing in the paratenic hosts.

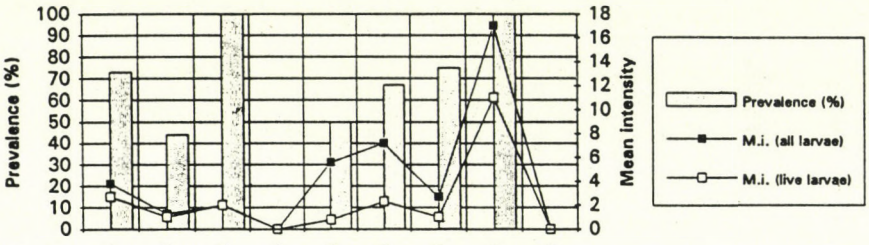


	Samplings	Nr. of fish examined
1	24.09.1991. Keszthely	4
2	02.10.1991. Almádi	3
3	07.11.1991. Csopak	1
4	11.03.1992. Keszthely	14
5	21.04.1992. Keszthely	8
6	20.05.1992. Tihany	1
7	24.06.1992. Keszthely	3
8	03.08.1992. Tihany	6
9	10.08.1992. Csopak	3
10	19.04.1993. Keszthely	1
11	05.07.1993. Tihany	1
12	09.08.1993. Csopak	3
13	01.09.1993. Kenese	16
14	21.10.1993. Keszthely	1
	Total examined	65

Chart 7. Prevalence and mean intensity of *Anguillicola crassus* larval infection of bitterling (*Rhodeus sericeus*) in Lake Balaton

A survey of the dynamics of infection during a 6-year period has already been conducted in The Netherlands as well (Haenen et al., 1994). In that study, however, the only paratenic host monitored was the smelt (*Osmerus eperlanus*), and exclusively the larvae occurring in the swimbladder were examined. Although the prevalence of infection of the smelt dropped below 40% after the first two years of the study and remained on that level thereafter, that set of data is not comparable with data from the 3-year study of larval infection of paratenic hosts from Lake Balaton, as the latter study involved an examination of the entire abdominal content of the fish. In addition, only a minority of larvae occurred in the swimbladder, while the majority were present in the principal predilection site, on the outer surface of the intestinal wall. The low mean intensity values (1.2–2.16) recorded in smelt by Haenen et al. (1994) also indicate that they found only part of the larvae occurring in a given fish specimen. In contrast to the present study, however, in some cases Haenen et al. (1994) detected also 4th stage larvae (L4), a

stage which occurs exclusively in the swimbladder. Like in cyprinids of Lake Balaton, in some instances the death of larvae following encapsulation by the host organism was observed also in smelt.



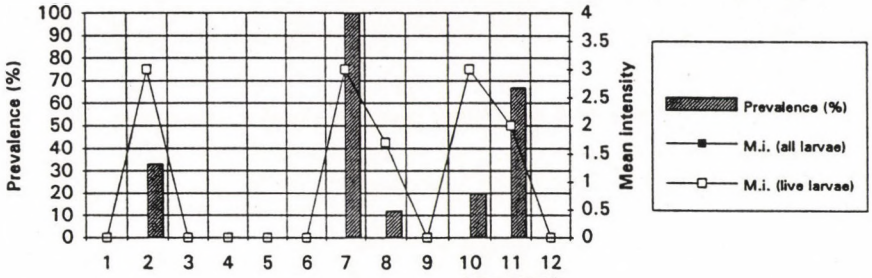
Samplings		Nr. of fish examined
1	16.09.1991. Keszthely	15
2	24.10.1991. Keszthely	9
3	02.10.1991. Almádi	2
4	07.11.1991. Csopak	1
5	11.03.1992. Keszthely	20
6	21.04.1992. Keszthely	9
7	24.06.1992. Keszthely	4
8	09.09.1992. Kenese	2
9	21.10.1993. Keszthely	1
	Total examined	63

Chart 8. Prevalence and mean intensity of *Anguillicola crassus* larval infection of Chinese rasbora (*Pseudorasbora parva*) in Lake Balaton

In an earlier study involving the examination of the swimbladder and the intestine, Haenen and van Banning (1990) found larvae exclusively in the swimbladder. Interestingly, 4th stage larvae occurred in the swimbladder of smelt, ruffe, perch and three-spined stickleback on several occasions, and in the ruffe, perch and three-spined stickleback even infection by pre-adult larval stages was recorded. In our 3-year study (which involved much higher numbers of species and specimens), L4 or pre-adult larvae could never be detected in paratenic hosts. The larvae detected always proved to be L3. In the same way, during their studies on fish from Lake Velence, a lake situated close to Lake Balaton, Pazooki and Székely (1994) could detect exclusively L3 in the paratenic hosts.

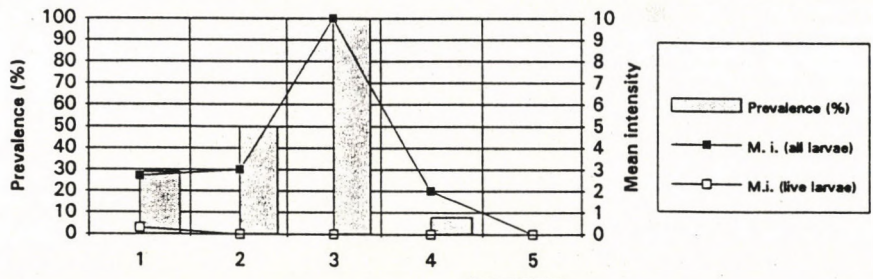
Certain Belgian (Thomas and Ollevier, 1992) and Dutch (Haenen, 1995) authors observed in several cases that *Anguillicola* larvae can develop and reach the L4 stage in some fish species other than the eel. These fish species, however, cannot be considered paratenic hosts, as larval development has taken place in them; nor can they be regarded as hosts as the helminths developing in them have failed to reach the imago stage. They can be designated "inadequate hosts". Dur-

ing this study, exclusively L3 were found in all fish examined; therefore, these species can consistently be regarded as paratenic hosts.



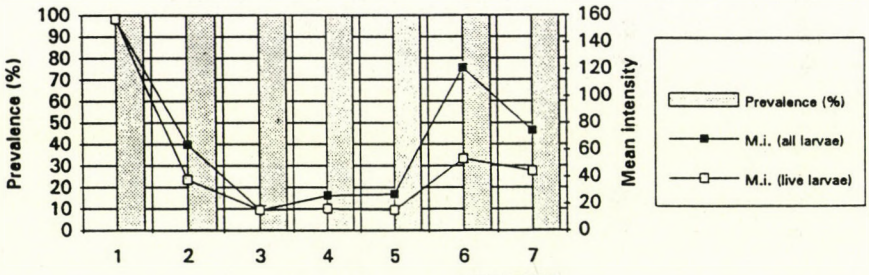
		Nr. of fish examined
1	05.09.1991. Tihany	1
2	07.11.1991. Csopak	3
3	24.06.1992. Keszthely	5
4	03.08.1992. Tihany	1
5	25.08.1992. Keszthely	4
6	09.09.1992. Kenese	4
7	19.04.1993. Keszthely	1
8	24.06.1993. Kenese	26
9	25.06.1993. Szabadi	4
10	05.07.1993. Tihany	5
11	23.08.1993. Keszthely	3
12	01.09.1993. Kenese	1
	Total examined	58

Chart 9. Prevalence and mean intensity of *Anguillicola crassus* larval infection of pike perch (*Stizostedion lucioperca*) in Lake Balaton



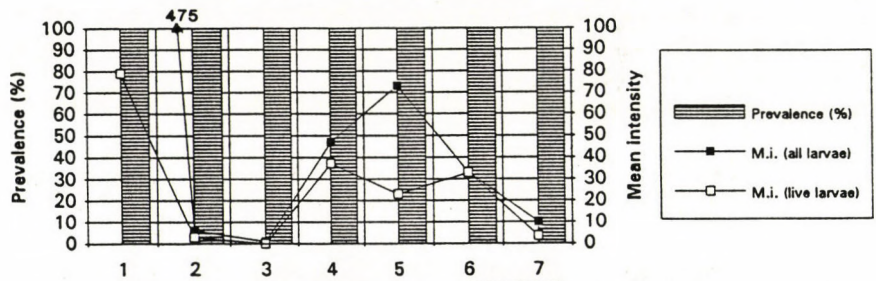
		Nr. of fish examined
1	11.03.1992. Keszthely	21
2	09.09.1992. Kenese	2
3	19.04.1993. Keszthely	1
4	23.08.1993. Keszthely	13
5	12.10.1993. Tihany	1
	Total examined	38

Chart 10. Prevalence and mean intensity of *Anguillicola crassus* larval infection of bream (*Abramis brama*) in Lake Balaton (1992–1993)



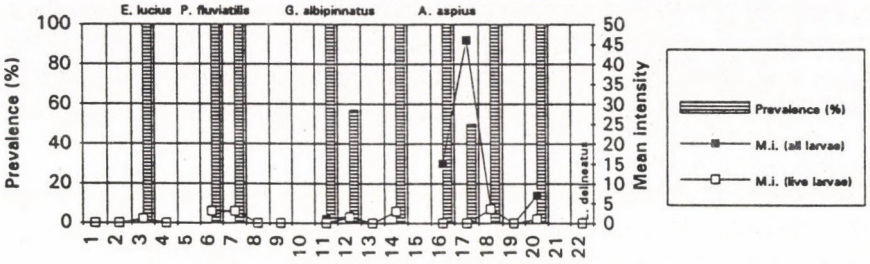
		Nr. of fish examined
1	24.10.1991. Keszthely	1
2	07.11.1991. Csopak	13
3	31.03.1992. Tihany	1
4	20.05.1992. Tihany	1
5	09.09.1992. Kenese	1
6	26.04.1993. Kenese	3
7	01.09.1993. Kenese	5
	Total examined	25

Chart 11. Prevalence and mean intensity of *Anguillicola crassus* larval infection of ruffe (*Gymnocephalus cernua*) in Lake Balaton



		Nr. of fish examined
1	16.09.1991. Keszthely	5
2	07.11.1991. Csopak	1
3	31.03.1992. Csopak	1
4	09.09.1992. Kenese	4
5	06.04.1993. Csopak	1
6	09.06.1993. Keszthely	1
7	01.09.1993. Kenese	2
	Total examined	15

Chart 12. Prevalence and mean intensity of *Anguillicola crassus* larval infection of catfishes (*Silurus glanis* and *Ictalurus nebulosus*) in Lake Balaton

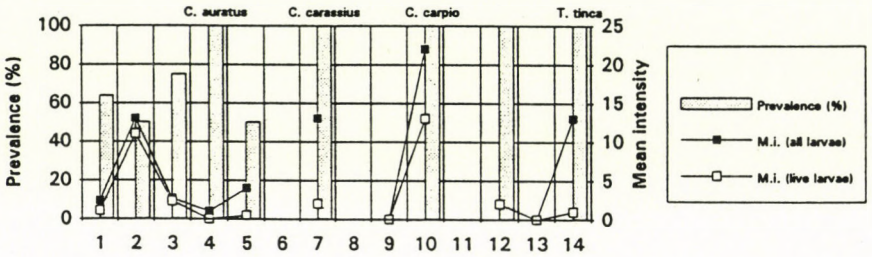


	Samplings	Nr. of fish examined
	Esox lucius	
1	07.11.1991. Csopak	4
2	03.08.1992. Tihany	2
3	10.08.1992. Csopak	1
4	05.07.1993. Tihany	1
	Total examined	8
	Perca fluviatilis	
6	17.10.1991. Keszthely	1
7	11.03.1992. Keszthely	1
8	21.04.1992. Keszthely	1
9	05.07.1993. Tihany	2
	Total examined	5
	Gobio albipinnatus	
11	17.09.1991. Keszthely	1
12	25.06.1993. Szabadi	14
13	05.07.1993. Tihany	1
14	01.09.1993. Kenese	1
	Total examined	17
	Aspius aspius	
16	11.03.1992. Keszthely	1
17	21.04.1992. Keszthely	2
18	24.06.1992. Keszthely	2
19	05.07.1993. Keszthely	1
20	21.10.1993. Keszthely	1
	Total examined	7
	Leucaspius delineatus	
22	11.03.1992. Keszthely	1

Chart 13. Prevalence and mean intensity of *Anguillicola crassus* larval infection in paratenic host fishes (*Esox lucius, Perca fluviatilis, Gobio albipinnatus, Aspius aspius, Leucaspius delineatus*) in Lake Balaton

Thomas and Ollevier (1992) studied the *Anguillicola* larval infection of paratenic host fish species in a Belgian channel over a one-year period. They found that in the warm period (from May to October) the prevalence of infection was in most species higher than in the cold season. From this fact they infer that fish contract the infection primarily in the summer. Although this hypothesis may seem logical, during our 3-year study of Lake Balaton fishes no seasonal fluctuation was found in the prevalence of infection.

The results of this study indicate that the L3 of *A. crassus* are extremely widespread in Lake Balaton, and most fish species of the lake serve as their paratenic hosts. The study also calls attention to the dissimilar nature of the host reaction in the different paratenic hosts. From the examinations the injury caused to the paratenic hosts by the pathological lesions observed cannot be assessed; at the same time, highly intensive infection of the European catfish may occasionally be of fatal outcome.



Samplings		Nr. of fish
	Carassius auratus gibelio	examined
1	02.10.1991. Almádi	11
2	07.11.1991. Csopak	2
3	24.06.1992. Keszthely	4
4	09.09.1992. Szabadi	1
5	09.09.1992.Kenese	4
	Total examined	22
6	*Carassius carassius*	
7	21.10.1993. Keszthely	1
8	*Cyprinus carpio*	
9	21.04.1992. Keszthely	1
10	24.10.1991. Keszthely	2
	Total examined	3
11	*Tinca tinca*	
12	02.10. 1991. Almádi	1
13	07.11.1991. Csopak	1
14	24.06.1992. Keszthely	1
	Total examined	3

Chart 14. Prevalence and mean intensity of *Anguillicola crassus* larval infection of carps (*Carassius auratus, Carassius auratus gibelio, Cyprinus carpio, Tinca tinca*) in Lake Balaton

Table 2

Anguillicola larval infection of paratenic host fish species caught in a large number of specimens in the three regions of Lake Balaton between 1991 and 1993 (ranges of the means calculated for each sampling)

Fish species	Number examined	Larvae recovered		
		prevalence, ranges	live larvae, ranges	all larvae, ranges
Western basin				
Alburnus alburnus	110	43–100	0–22	4–25.3
Lepomis gibbosus	58	43–100	1–5.6	1–5.6
Neogobius fluviatilis	49	63–100	4.0–22	5.5–22.3
Rutilus rutilus	85	13–86	0–19	2–39.5
Central basin				
Alburnus alburnus	170	20–100	0.2–3.9	3–12.3
Lepomis gibbosus	65	0–75	0–3.3	0–3.3
Neogobius fluviatilis	83	60–100	1–14.2	2–14.8
Rutilus rutilus	49	0–100	0–1.3	0–31
Eastern basin				
Alburnus alburnus	98	20–100	0–1.5	3.6–15.6
Lepomis gibbosus	75	30–80	1.7–3.0	1.7–3.8
Neogobius fluviatilis	37	70–100	8–9.6	9–10.2
Rutilus rutilus	26	50–94	0.3–37	5.2–38

Table 3

Anguillicola larval infection of paratenic host fish species caught in a small number of specimens in Lake Balaton between 1991 and 1993 (ranges of the means calculated for each sampling)

Fish species	Number examined	Larvae recovered		
		prevalence, ranges	live larvae, ranges	all larvae, ranges
Blicca bjoerkna	78	0–100	0–1.5	0–20
Scardinius erythrophthalmus	68	0–100	0–2.8	0–68
Rhodeus sericeus amarus	65	0–100	0–0.4	0–10.3
Pseudorasbora parva	63	0–100	0–11	0–17
Stizostedion lucioperca	58	0–100	0–3	0–3
Abramis brama	38	0–100	0–0.3	0–10
Gymnocephalus cernua	25	100	15–157	15–157
Silurus glanis	14	100	0–79	1–475
Ictalurus nebulosus	1	100	32	32
Esox lucius	8	0–100	0–1	0–1
Perca fluviatilis	5	0–100	0–3	0–3
Gobio albipinnatus	17	0–100	0–3	0–3
Aspius aspius	7	0–100	0–3.5	0–6
Leucaspius delineatus	1	0	0	0
Carassius carassius	1	100	2	13
Carassius auratus gibelio	22	50–100	0–11	1.0–13
Cyprinus carpio	3	0–100	0–13	0–22
Tinca tinca	3	0–100	0–2	0–13

Acknowledgements

The author wishes to thank the staff of the Balaton Limnological Research Institute of the Hungarian Academy of Sciences for making available their research boat for the fishing trips. Thanks are due to the crew of the boat, especially to István Báthory, for their help in the fish collections. The author is indebted to Dr. Kálmán Molnár, head of the Fish Pathology Team (Veterinary Medical Research Institute, Hungarian Academy of Sciences) for valuable advice given during this work, and to all other members of the team for their work in the collection and processing of fish, especially to Mrs. Emese Papp and Miss Andrea Jávori who rendered the biggest help in that work. Thanks are also due to Mrs. Margit Székely for her help in collecting fish.

This work was supported by the National Research Fund (OTKA), project no. T 6035.

References

Belpaire, C., de Charleroy, D., Thomas, K., van Damme, P. and Ollevier F. (1989): Effect of restocking on the distribution of the swimbladder nematode *Anguillicola crassus* in Flanders, Belgium. J. Appl. Ichthyol. **5,** 151–153.

Boon, J. H., Lokin, C. J. A., Ceusters, R. and Ollevier, F. (1989): Some properties of the blood of European eel (*Anguilla anguilla*) and the possible relationship with *Anguillicola crassus* infestations. Aquaculture **76,** 203–208.

Boon, J. H., Augustijn, H., Cannaerts, V. M. H., Lokin, C. J. A., Machiels, M. A. M. and Ollevier, F. (1990*a*): The suitability of experimental inoculations with infective larvae of *Anguillicola crassus* and their effect on the growth and mortality of the European eel (*Anguilla anguilla*). Aquaculture **87,** 111–120.

Boon, J. H., Cannaerts, V. M. H., Augustijn, H., Machiels, M. A. M., De Charleroy, D. and Ollevier, F. (1990*b*): The effect of different infection levels with infective larvae of *Anguillicola crassus* on haematological parameters of European eel (*Anguilla anguilla*). Aquaculture **87,** 243–253.

Boon, J. H., Zuxu, Y. and Booms, G. H. R. (1990*c*): Effects on *Anguillicola crassus* and *Trypanosoma granulosum* infection on peripheral blood cells of European eel (*Anguilla anguilla* L.). Bull. Eur. Ass. Fish Pathol. **10,** 143–145.

Buchmann, K., Pedersen, L. O. and Glamann, J. (1991): Humoral immune response of European eel *Anguilla anguilla* to a major antigen in *Anguillicola crassus* (Nematoda). Dis. aquat. Org. **12,** 55–57.

Cannaerts, V. (1989): Interactie van *Anguillicola crassus*, met enkele typishe reservoirgastheren en de eindgastheer, *Anguilla anguilla*. M.Sc. Dissertation, Catholic University of Leuven, Belgium.

De Charleroy, D., Grisez, L., Thomas, K., Belpaire, C. and Ollevier, F. (1990): The life cycle of *Anguillicola crassus*. Dis. aquat. Org. **8,** 77–84.

Dupont, F. and Petter, A. J. (1988): *Anguillicola*, une épizootie plurispécifique en Europe. Apparition d'*Anguillicola crassa* (Nematoda, Anguillicolidae) chez l'Anguille europeene (*Anguilla anguilla*) en Camargue, Sud de la France. Bull. Fr. Péche Piscic. **308,** 38–41.

Haenen, O. L. M. and van Banning, P. (1990): Detection of larvae of *Anguillicola crassus* (an eel swimbladder nematode) in freshwater fish species. Aquaculture **87,** 103–109.

Haenen, O. L. M. and van Banning, P. (1991): Experimental transmission of *Anguillicola crassus* (Nematoda, Dracunculoidea) larvae from infected prey fish to the eel, *Anguilla anguilla*. Aquaculture **92,** 115–119.

Haenen, O. L. M., Grisez, L., De Charleroy, D., Belpaire, C. and Ollevier, F. (1989): Experimentally induced infections of European eel *Anguilla anguilla* with *Anguillicola crassus* (Nematoda, Dracunculoidea) and subsequent migration of larvae. Dis. aquat. Org. **7,** 97–101.

Haenen, O. L. M., van Banning, P. and Dekker, W. (1994): Infection of eel *Anguilla anguilla* (L.) and smelt *Osmerus eperlanus* (L.) with *Anguillicola crassus* (Nematoda, Dracunculoidea) in the Netherlands from 1986–1992. Aquaculture. **126,** 219–229.

Haenen, O. L. M. (1995): *Anguillicola crassus* (Nematoda, Dracunculoidea) infections of European eel *(Anguilla anguilla)* in the Netherlands: epidemiology, pathogenesis and pathobiology. PhD dissertation, DLO-Institute for Animal Sci. and Health, Lelystad, The Netherlands.

Hartmann, S. (1987): Schwimblassenwürmer beim Aal. Fischer & Teichwirt **38,** 2–3.

Höglund, J. and Andersson, J. (1993): Prevalence and abundance of *Anguillicola crassus* in the European eel (*Anguilla anguilla*) at a thermal discharge site on the Swedish coast. J. Appl. Ichthyol. **9,** 115–122.

Höglund, J., Andersson, J. and Hardig, J. (1992): Haematological responses in the European eel, *Anguilla anguilla* L., to sublethal infestation by *Anguillicola crassus* in a thermal effluent of the Swedish Baltic. J. Fish Dis. **15,** 507–514.

Höglund, J. and Pilström, L. (1994): Purification of adult *Anguillicola crassus* whole-worm extract antigens for detection of specific antibodies in serum from the European eel *(Anguilla anguilla)* by Elisa. Fish Shellfish Immunol. **4,** 311–319.

Höglund, J. and Thomas, K. (1992): The black goby, *Gobius niger*, as a potential paratenic host for the parasitic nematode *Anguillicola crassus* in a thermal effluent of the Baltic. Dis. aquat. Org. **13,** 175–180.

Kamstra, A. (1990): Anguillicola in Dutch eelfarms; current state. Int. Revue ges. Hydrobiol. **75,** 867–874.

Kennedy, C. R. and Fitch, D. J. (1990): Colonization, larval survival and epidemiology of the nematode *Anguillicola crassus,* parasitic in the eel, *Anguilla anguilla*, in Britain. J. Fish Biol. **36,** 117–131.

Koops, H. and Hartmann, F. (1989): *Anguillicola* infestations in Germany and in German eel imports. J. Appl. Ichthyol. **1,** 41–45.

Køie, M. (1991): Swimbladder nematodes (*Anguillicola* spp.) and gill monogeneans (*Pseudodactylogyrus* spp.) parasitic on the European eel (*Anguilla anguilla*). J. Cons. int. Explor. Mer. **47,** 391–398.

Liewes, E.W. and Schaminee-Main, S. (1987): Onderzoek naar de effecten van de parasiet *Anguillicola crassa* op de ontwikkeling van de paling (*Anguilla anguilla*) in een zout water palingmesterij. Internal report, Texvis B.V., Den Burg Texel, The Netherlands.

Molnár, K., Baska, F., Csaba, Gy., Glávits, R. and Székely, Cs. (1993): Pathological and histopathological studies of the swimbladder of eels *Anguilla anguilla* infected by *Anguillicola crassus* (Nematoda: Dracunculoidea). Dis. aquat. Org. **15,** 41-50.

Molnár, K., Székely, Cs. and Baska, F. (1991): Mass mortality of eel in Lake Balaton due to *Anguillicola crassus* infection. Bull. Eur. Ass. Fish Pathol. **11,** 211–212.

Molnár, K., Székely, Cs. and Perényi, M. (1994): Dynamics of *Anguillicola crassus* (Nematoda: Dracunculoidea) infection in eels of Lake Balaton, Hungary. Folia Parasitol. **41,** 193–202.

Moravec, F. (1992): Spreading of the nematode *Anguillicola crassus* (Dracunculoidea) among eel populations in Europe. Folia Parasitol. **39,** 247–248.

Möller, H., Holst, S., Lüchtenberg, H. and Petersen, F. (1991): Infection of eel *Anguilla anguilla* from the River Elbe estuary with two nematodes, *Anguillicola crassus* and *Pseudoterranova decipiens*. Dis. aquat. Org. **11,** 193–199.

Neumann, W. (1985). Schwimmblasenparasit *Anguillicola* bei Aalen. Fischer und Teichwirt, 36: 322.

Paggi, L., Orecchia, P., Minervini, R. and Mattiucci, S. (1982): Sulla comparsa di *Anguillicola australiensis* Johnston e Mawson, 1940 (Dracunculoidea: Anguillicolidae) in *Anguilla anguilla* del Lago di Bracciano. Parassitologia **24,** 139–144.

Pazooki, J. and Székely, Cs. (1994): Survey of the paratenic hosts of *Anguillicola crassus* in Lake Velence, Hungary. Acta Vet. Hung. **42,** 87–97.

Peters, G. and Hartmann, F. (1986): *Anguillicola*, a parasitic nematode of the swim bladder spreading among eel populations in Europe. Dis. aquat. Org. **1,** 223–230.

Sarti, M., Georgetti, G. and Brisinelle, W. (1985): A new problem for intensive eel rearing in Italy: *Anguillicola australiensis*. Proc. E.A.F.P. Congress, Montpellier, September 1985, p. 95.

Sprengel, G. and Lüchtenberg, H. (1991): Infection by endoparasites reduces maximum swimming speed of Europaen smelt *Osmerus eperlanus* and European eel, *Anguilla anguilla*. Dis. aquat. Org. **11,** 31–35.

Székely, Cs. (1994): Paratenic hosts of the parasitic nematode *Anguillicola crassus* in Lake Balaton, Hungary. Dis. aquat. Org. **18,** 11–20.

Székely, Cs., Láng, M. and Csaba, Gy. (1991): First occurrence of *Anguillicola crassus* in Hungary. Bull. Eur. Ass. Fish Pathol. **11,** 162–163.

Taraschewski, H., Moravec, F., Lamah, T. and Anders, K. (1987): Distribution and morphology of two helminths recently introduced into European eel populations: *Anguillicola crassus* (Nematoda, Dracunculoidea) and *Paratenuisentis ambiguus* (Acanthocephala, Tenuisentidae). Dis. aquat. Org. **3,** 167–176.

Thomas, K. and Ollevier, F. (1992): Population biology of *Anguillicola crassus* in the final host *Anguilla anguilla*. Dis. aquat. Org. **14,** 163–170.

van Banning, P. and Haenen, O. L. M. (1990): Effects of the swimbladder nematode *Anguillicola crassus* in wild and farmed eel, *Anguilla anguilla*. In: Perkins, F. O. and Cheng, T. C. (eds) Pathology in Marine Science, Academic Press, New York, pp. 317–330.

van Willigen, J. and Dekker, W. (1989): 1988 update on *Anguillicola* in Dutch outdoor waters. EIFAC (FAO), Working Party on Eel. Oporto, Portugal, 29 May to 3 June, 1989, 8 pp.

Acta Veterinaria Hungarica 43 (4), pp. 423–429 (1995)

EVALUATION OF BLOOD LIPID PEROXIDATION PARAMETERS IN CARBON TETRACHLORIDE (CCl_4) TOXICITY IN SHEEP

P. VAJDOVICH, A. SZILÁGYI and T. GAÁL

Department of Internal Medicine, University of Veterinary Science, H–1400 Budapest, P.O. Box 2, Hungary

(Received April 5, 1995)

Seven female, 2-year-old, nonpregnant, Merino ewes were treated with a nonlethal dose of 0.3 ml/kg body mass carbon tetrachloride (CCl_4) in 1:1 v/v dilution with paraffin oil via a stomach tube into the rumen. Blood samples were collected one day before and on the first, second, third, seventh and tenth day after toxin exposure to study the changes of the lipid peroxidation (LP) status of red blood cell haemolyzate (RBC-haem). The severity of liver damage was monitored by determination of aspartate aminotransferase (AST) activity and bilirubin concentration in the blood plasma. Twenty-four h after CCl_4 exposure all animals became lethargic and anorexic, their heart rate and respiratory rate increased. On the subsequent two days these signs became more severe, but by the 10th day the symptoms disappeared. On the 1st and 2nd day following CCl_4 exposure the concentration of malondialdehyde (MDA) — an end product of LP — in RBC-haem significantly increased. A slight decrease was found on the 3rd, 7th and 10th day, but MDA values remained significantly higher than the basal ones. The activity of glutathione peroxidase (GPX) in RBC-haem increased slowly on the 1st and 2nd day, then it rose intensively on the third day. GPX activity remained elevated until the 7th day, but on the 10th day it dropped again. Catalase (Cat) activity in RBC-haem did not show any significant changes during the experiment. AST activity in blood plasma showed a two-fold increase in the first three days; later on the high values decreased. Total and direct plasma bilirubin concentration slightly increased on the 3rd day, then both decreased. LP effects in CCl_4-induced hepatocellular injury were significant in sheep, in line with the results of experiments on other species such as rats. The LP effects were demonstrated by the elevated MDA concentration and GPX activity.

Key words: Sheep, carbon tetrachloride, lipid peroxidation, blood, liver

Carbon tetrachloride (CCl_4) poisoning previously occurred in animals mainly as a result of the (mis)use of this substance as an anthelmintic, and it usually caused acute disease. The severe clinical symptoms include a loss of appetite, gastrointestinal pain followed by diarrhoea, and liver injury often accompanied by signs of icterus (Setchell, 1962). Nowadays, CCl_4 is one of the compounds most

0236–6290/95/$ 5.00 © 1995 *Akadémiai Kiadó, Budapest*

commonly used for inducing experimental liver injury (Di Luzio, 1973; Slater et al., 1985; Lettéron et al., 1990). It is a hepatotoxic drug which induces zonal centrolobular necrosis followed by fatty liver degeneration and liver cirrhosis (Di Luzio, 1973).

Numerous studies have demonstrated that certain types of chemical-induced liver injury such as carbon tetrachloride toxicity may involve the peroxidation of subcellular lipids (Di Luzio, 1968; Di Luzio and Hartman, 1969; Di Luzio, 1973). Data have also been presented to suggest that the primary event in the development of the ethanol-induced fatty liver, as well as in liver injuries induced by other chemicals, is the formation of lipoperoxides at selective subcellular sites because of the alteration in antioxidant activity of the hepatic cell (Di Luzio, 1968; Di Luzio and Hartman, 1969; Di Luzio, 1973). Enhanced lipid peroxidation mediated by carbon tetrachloride is due initially to the formation of the trichloromethyl free radical ($CCl_3\cdot$). This radical (or even more its peroxyl derivative, $CCl_3OO\cdot$) may abstract a hydrogen atom from unsaturated fatty acids and/or may covalently bind to lipids and proteins (Labbe et al., 1987). Similarly, when CCl_4 is replaced by halothane, the corresponding peroxyl radical, $CF_3CHClOO\cdot$ is formed. In both cases these free radical intermediates are involved in the hepatotoxicity of the compounds (Recknagel and Glende, 1973). Abstraction of hydrogen atoms from unsaturated fatty acids by such radicals create carbon-centred lipid radicals (McCay et al., 1984). These lipid radicals quickly accept molecular oxygen to form lipid peroxyl radicals, thereby initiating the process of lipid peroxidation. Unless neutralized by vitamin E or other radical scavengers, these lipid peroxyl radicals in turn abstract hydrogen atoms from other lipid molecules, thereby propagating the process of lipid peroxidation (Labbe et al., 1987).

The connections between liver diseases and free radicals in certain circumstances in humans and rats are well documented (Muth, 1960; Barker et al., 1969; Di Luzio, 1973). A report has shown that sheep fed a low selenium diet are increasingly susceptible to CCl_4-induced liver degeneration (Muth, 1960). Using this CCl_4 could give some information about other chemical-induced toxic liver damages (such as halothane) in ruminants. Results obtained from such an experiment can provide basal data for further studies.

The development of elevated lipid peroxidation (LP) processes in an organism can be easily followed by clinico-chemical methods measuring some characteristic parameters of LP such as scavengers, numerous enzymes, end-products etc. in the blood or other tissues. Despite this there is an apparent scarcity of investigations of measured blood parameters of LP and their changes in ruminants in the course CCl_4 toxicosis. Therefore, in the present study we have determined some frequently studied blood parameters of LP and their changes during mild carbon tetrachloride toxicity in sheep.

Materials and methods

Animals and treatments. Seven nonpregnant Merino ewes, two to three years old, were housed individually and treated with 0.3 ml/kg body mass carbon tetrachloride in 1:1 v/v dilution with paraffin oil via a stomach tube into the rumen. Before, during and after the trial the same diet was given, which consisted of alfalfa hay, pelleted concentrate and was supplemented with an appropriate amount of minerals and fresh water *ad libitum.*

Blood sampling and preparation. Blood samples were collected from a jugular vein into heparinised tubes at 8:00 a.m. before and on the 1st, 2nd, 3rd, 7th and 10th day following the CCl_4 exposure. Samples were centrifuged at 2000 rpm for 10 min. The sedimented cells were separated from the plasma and washed and recentrifuged three times with 0.9% sodium chloride solution. Freshly washed erythrocytes were haemolyzed with a nine-fold volume of distilled water.

Analytical procedures. In the red blood cell haemolyzate (RBC-haem), the concentrations of total protein (TP) and thiobarbituric acid reactive substances (TBARS) were determined along with the enzyme activities of glutathione peroxidase (GPX, E.C. 1.11.1.9.) and catalase (Cat, E.C. 1.11.1.6.). TP was measured photometrically using Folin-phenol reagent (Lowry et al., 1952). TBARS were measured according to Placer et al. (1966) using 1,1,3,3-tetramethoxypropane (malonaldehyde bis[dimethyl acetal]) as standard, and the results were expressed as malondialdehyde (MDA). Reduced glutathione, cumene hydroperoxide and Ellman's reagent were used for determination of GPX, as described by Lawrence and Burk (1976). Catalase (Cat, E.C. 1.11.1.6.) enzyme activity was assayed by the method described by Beers and Sizer (1952), in which the loss of hydrogen peroxide was followed spectrophotometrically. Catalase activity was expressed in Bergmeyer Units (BU), means the activity of the enzyme catabolyzing 1 μmol H_2O_2 in one minute at 25 °C (14). GPX and Cat activities were referred to 1 g total protein of RBC-haem.

Aspartate aminotransferase (AST [GOT], E.C. 2.6.1.1.) activity of the plasma was measured colorimetrically by the method of Reitman and Frankel (1957). Total bilirubin and direct bilirubin concentrations were assayed by the diazo-method described by Walters and Gerade (1970).

Statistical analysis. Individual results were used for biometric calculations by analysis of variance (ANOVA), with the help of a SAS/SAT computer programme. The results either given in table or illustrated graphically show the mean and the standard error (SEM). Only results found to be significantly different from the basal values are marked.

Results

Twenty-four h after CCl_4 exposure the animals became lethargic and anorexic with increased heart and respiratory rates and pale mucous membranes. Clinical signs changed in parallel with the clinico-pathological findings of mild toxicosis. Following the 10-day trial all sheep recovered clinically.

Laboratory results found in the plasma are shown in Table 1. AST activity showed a two-fold increase on the first, second and third day, whilst by the end of the experiment these high values decreased. There were no significant changes in TP and bilirubin results, but both total and direct bilirubin concentrations increased, then both decreased.

Table 1

Blood serum (plasma) results in sheep treated with carbon tetrachloride (n = 7; means ± SEM)

Variable	Basal values	Days				
		1	2	3	7	10
Total protein, g/l	74.3 ±8.7	76.7 ±8.3	75.5 ±7.8	74.4 ±7.2	69.5 ±6	65.2 ±6.9
Aspartate aminotransferase, U/l	54 ±9	125* ±31	130* ±46	110* ±33	87* ±26	66 ±20
Total bilimbin, μmol/l	5.2 ±1.3	5.4 ±1.1	5.8 ±1.5	7.1 ±1.4	6 ±1.3	4.6 ±1.4
Direct bilimbin, μmol/l	1.2 ±0.3	2.1 ±0.6	2.1 ±0.6	2.4 ±0.7	2.2 ±0.6	1.7 ±0.7

* = probability of difference from the basal value $P < 0.05$

Changes in MDA concentration and GPX activities are shown in Fig. 1. Following CCl_4 exposure a steady increase of both components was found, but the rate of increase of MDA somewhat exceeded that of GPX.

Cat enzyme activity in RBC-haem did not show any significant changes during the experiment and varied between 20–26 BU/g protein.

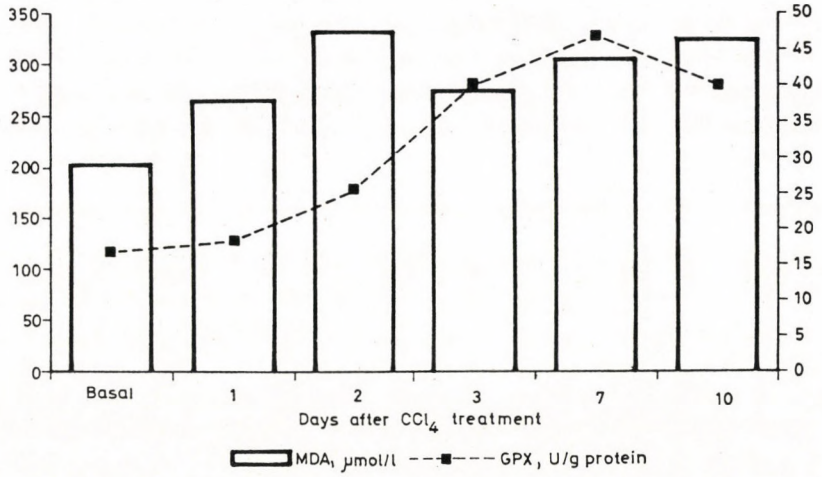


Fig. 1. Concentration of TBARS expressed as MDA and GPX activity in RBC haemolysate

	Basal	Day 1	Day 2	Day 3	Day 7	Day 10
MDA	204	*266	*332	*275	*305	*324
	±26.4	±5.8	±25.3	±20.4	±31.4	±27.3
GPX	16.6	18.3	*25.4	*40	*47	*40.2
	±2.2	±3.7	±7.0	±9.4	±12.8	±2.4

Probability of difference from the basal value marked with *:$P < 0.05$

Discussion

The toxic effect of carbon tetrachloride in sheep manifested itself in clinical signs (lethargy, anorexia, pale mucous membranes) and in an increased activity of AST. Regarding the clinical signs of toxicity, our observations agree with those of Muth (1960). As to AST, our results differ from those of Kondos and McClymont (1965) who recorded a higher activity (700 U/l), while the maximum activity found by us was around 130 U/l 24 h after drug administration. This difference could be explained by the different dose, frequency and route of CCl_4 application and the laboratory methods used. Increased AST activity and the elevation of total and direct bilirubin concentrations were due to the hepatocellular injury.

Elevation of MDA concentration reflected the higher production of lipid peroxyl radicals from the peroxidation of polyunsaturated fatty acids (PUFAs). As cell and subcellular lipoprotein membranes are rich in PUFAs, these should be seriously injured by the increased peroxidative effect of free radicals generated from

CCl_4. Increased GPX activity of the red blood cell haemolyzate could be interpreted as an enhanced response of the enzymatic defence mechanism in the organism, the enyzme being a member of this antioxidant defence system. Such an effect was not observed in the activity of catalase. In fasted sheep Gaál et al. (1993) observed increases in both the level of MDA and the activity of catalase. This shows that elevated LP processes in an organism can be accompanied by different effects on enzyme activities.

Further studies should be performed to investigate the role of LP and free radical formation in cases of other liver disorders in ruminants, especially those of metabolic origin. In addition it is necessary to investigate the potential to extend the so-called "liver protective therapy" with the application of antioxidants such as N,N-diphenyl-p-phenylendiamine, silymarin or vitamin E, as they have been reported in rats and mice (Hove, 1949; Di Luzio, 1973; Lettéron et al., 1990).

In conclusion, a mild CCl_4 toxicosis caused significant changes in LP processes of sheep. It is suspected that similar changes may occur in other types of toxic (especially chemical-induced) liver injury of ruminants where liver necrosis develops.

References

Barker, E. A., Arccasoz, M. and Smucler, E. A. (1969): A comparison of the effects of partial surgical and partial chemical (CCl_4) hepatectomy on microsomal cytochrome b5 and P450 and oxidative N-demethylation. Agents Actions **1**, 27–34.

Beers, R. F. and Sizer, I. W. (1952): Spectrophotometric method for measuring the breakdown of hydrogen peroxide by catalase. J. Biol. Chem. **195**, 133–140.

Di Luzio, N. R. (1968): Letter to the editor: The role of lipid peroxidation and antioxidants in ethanol-induced lipid alterations. Exptl. Mol. Pathol. **8**,. 394–402.

Di Luzio, N. R. (1973): Antioxidants, lipid peroxidation and chemical-induced liver injury. Fed. Proc. **32**, 1875–1881.

Di Luzio, N. R. and Hartman, A. D. (1969): The effect of ethanol and carbon tetrachloride administration on hepatic lipid-soluble antioxidant activity. Exptl. Mol. Pathol. **11**, 38–52.

Gaál, T., Mézes, M., Miskucza, O., Ribiczey-Szabó, P. (1993): Effect of fasting on blood lipid peroxidation parameters of sheep. Res. Vet. Sci. **55**, 104–107.

Hove, E. L. (1949): Comparison of fatal tocopherol deficiency disease in rats with the syndrome caused by CCl_4. Ann. New York Acad. Sci. **52**, 217–224.

Kondos, A. C. and McClymont, G. L. (1965): Enhanced toxicity of carbon tetrachloride in sheep on high protein intakes. Aust. Vet. J. **41**, 349–351.

Labbe, G., Descatoire, V., Lettéron, P., Degott, C., Tinel, M., Larrey, D., Carrion-Pavlov, Y., Genéve, J., Amouyal, G. and Pessayre, D. (1987): The drug methoxsalen, a suicide substrate for cytochrome P-450, decreases the metabolic activation, and prevents the hepatotoxicity of carbon tetrachloride in mice. Biochem Pharmacol. **36,** 907–914.

Lawrence, R. A. and Burk, R. F. (1976): Gluthatione-peroxidase activity in selenium-deficient rat liver. Biochem. Biophys. Res. Comm. **71**, 952–955.

Lettéron, P., Labbe, G., Degott, C., Berson, A., Fromenty, B., Delafroge, M., Larrey, D. and Pessayre, D. (1990): Mechanism for the protective effects of silymarin against carbon tetrachloride-induced lipid peroxidation and hepatotoxicity in mice. Biochem. Pharm. **39**, 2027–2034.

Lowry, O. H., Rosenbrough, N. J., Farr, A. L. and Randall, R. J. (1952): Protein measurement with the Folin-phenol reagent. J. Biol. Chem. **193**, 265–275.

McCay, P. B., Lai, E. K., Poyer, J. L., Dubose, C. M. and Janzen, E. G. (1984): Oxygen- and carbon-centered free radical formation during carbon tetrachloride metabolism. Observation of lipid radicals *in vivo* and *in vitro*. J. Biol. Chem. **259**, 2135–2143.

Muth, O. H. (1960): Carbon tetrachloride poisoning of ewes on a low selenium ration, Am. J. Vet. Res. **21**, 86–87.

Placer, Z. A., Cushman, L. and Johnson, B. C. (1966): Estimation of product of lipid peroxidation (malonyl dialdehyde) in biochemical systems. Anal. Biochem. **16**, 359–364.

Recknagel, R. O. and Glende, E. A. (1973): Chemical mechanisms in carbon tetrachloride toxicity. Crit. Rev. Toxicol. **38**, 263–270.

Reitman, S. and Frankel, S. (1957): A colorimetric method for the determination of serum glutamic oxalacetic and glutamic pyruvic transaminases. Am. J. Clin. Pathol. **28**, 56–63.

Setchell, B. P. (1962): Poisoning of sheep with anthelmintic doses of carbon tetrachloride: Chemical Pathology. Aust. Vet. J. **38**, 580–582.

Slater, T. F., Cheesman, K. H. and Ingold, K. U. (1985): Carbon tetrachloride toxicity as a model for studying free-radical mediated liver injury. Philos. Trans. R. Soc. London, Ser. B **311**, 633.

Walters, M. I. and Gerade, R. W. (1970): Ultramicromethod for the determination of the conjugated and total bilirubin in serum or plasma. Microchem. **15**, 231–235.

Acta Veterinaria Hungarica 43 (4), pp. 431–441 (1995)

EFFECTS OF HISTAMINE ON GIZZARD EROSIONS AND ON THE ACTIVITY OF SELECTED ENZYMES IN CHICKENS

P. DŽAJA[1], Ž. GRABAREVIĆ[2], J. PERIĆ[1], Branka ARTUKOVIĆ[2], Marina TIŠLJAR[3], V. MRLJAK[4] and Edita ŠOŠTARIĆ[5*]

[1]Department of Forensic Veterinary Medicine and [2]Department of General Pathology and Pathological Morphology, Veterinary Faculty, University of Zagreb, 41000 Zagreb, Heinzelova 55, Croatia; [3]Croatian Veterinary Institute, Poultry Center, Zagreb; [4]Clinic of Internal Diseases of Domestic Animals; [5]Veterinary Faculty, University of Zagreb

(Received February 1, 1995)

Dose- (5, 10 and 15 mg/kg body mass) and time-dependent (2, 6, 12 and 24 h after treatment) effects of histamine on gizzard lesions and serum AST, ALT and CK activities of chickens are reported. Morphometric results and histopathological examination revealed that the most effective histamine dose which induced severe gizzard lesions was 10 mg/kg b.m., especially 2, 6 and 12 h after administration. No difference from the control values was found after 24 h. That dose also induced an elevation of serum enzyme (AST, ALT, CK) activities, which was most expressed for the activity of ALT.

Key words: Histamine, gizzard erosion, AST, ALT, CK, chicken

Histamine, a vasoactive amine, is ubiquitous in the organism, mostly occurring in the skin, lungs, central nervous system and digestive tract. Besides its several other activities, histamine has an important role in enhancing gastric secretion and the secretory activity of exocrine glands. Although its involvement in hydrochloric acid and pepsin secretion is well known, the importance of histamine in the aetiology of stress-induced erosions and ulcerations has not been clarified yet. The literature contains many contradictory data on the ulcerogenicity of histamine. Paré and Glavin (1986) have reported numerous studies where some authors observed a protective activity of histamine on stress ulcuses. There are few studies describing the effects of histamine on gizzard erosion (GE), although many authors reported that the feeding of a large amount of fish meal, deteriorated or not, induces gizzard erosions and ulceration. At first, most authors regarded the histamine contained in fish meal as the main factor responsible for the lesions. Janssen (1971) reported that the feeding of a high concentration of fish meal from Peru in poultry feed was the main cause of the disease, Johnson and Piendo (1971)

*5th year student

0236–6290/95/$ 5.00 © 1995 *Akadémiai Kiadó, Budapest*

estimated that fish meal fed at a ratio of 12% or higher induces disease. Zorko (1974), Harry et al. (1975), Harry and Tucker (1976), Kubena et al. (1976), Horaguchi et al. (1980), Umemura et al. (1981), Itakura et al. (1981) and many others have come to the same conclusion.

Rinehart et al. (1976) observed that heat treatment (120–130 °C for 4–8 h) of fish meal induces gizzard erosions and ulceration. Their finding was confirmed by Hopkins et al. (1976) who observed that heated fish meal (120 °C) induces gastric lesions, while fish meal without heat treatment does not. Masumura et al. (1981) suggested that toxic products resulting from released free histamine and histidine reacting with fish proteins during heating might be responsible for inducing the lesions. Umemura (1982) reports that mackerel meal heated at 135 °C for 3 h induces gastric lesions while it is nontoxic if heated at 110 °C and 160 °C for the same duration, and he concludes that a toxic substance is formed at 135 °C and it is destroyed at 160 °C. Kuba et al. (1983) state that the quantity of histamine is not related to food toxicity before heating while that of histidine is. They report that the quantity of histamine and histidine depends on the quality of fish meal and that prolonged heating decreases the amount of histidine and increases that of histamine. Okazaki et al. (1983) stated that a recently discovered substance called "gizzerosine", isolated from mackerel meal, was responsible for gizzard erosions and ulcerations. Grabarević et al. (1993) also described an experimental model of stress-induced gizzard erosion in four-day-old chickens. The same authors pointed out the fact that in Croatia the majority of GE cases are observed in very young broilers as a consequence of inadequate transport and environmental conditions. On the contrary, Kántás and Dobos-Kovács (1990) reported that GE is a disease affecting older broilers and laying hens.

Based on data available in the literature it is clear that investigations into the dose- and time-related effects of a single dose of histamine are yet to be done. The aim of this work was to accurately demonstrate the effect of histamine on the gizzard and on the activity of some serum enzymes in healthy chicks.

Materials and methods

One hundred and seventy male Isa Brown chickens were used in this study. After three days of accommodation, on the fourth day of life the chicks were divided into five groups as follows: K0 (n = 10): healthy, untreated control; K1 (n = 40): chickens treated with 0.5 ml of saline; H1 (n = 40): chickens treated with 5 mg/kg b.m. of histamine; H2 (n = 40): chickens treated with 10 mg/kg b.m. of histamine; H3 (n = 40): chickens treated with 15 mg/kg b.m. of histamine.

Saline and the above doses of histamine (PLIVA, Zagreb) were administered in to the crop via plastic tubes. The treated animals (groups K1, H1, H2 and H3) were euthanatized under chloroform anesthesia at different intervals, in

groups of ten birds, 2, 6, 12 and 24 h after histamine or saline administration. Birds of group K0 (i.e. healthy chicks) were sacrificed simultaneously with the administration of histamine and saline. Blood serum samples were taken for biochemical analysis, and an assessment of all gizzard lesions was performed. All gizzards were cut open, rinsed in water, and visible lesions were transcribed on transparent plastic foil. Individual lesions were measured through a millimeter paper and their sum was taken as the total length of gizzard lesions.

For histopathological analysis all the samples were fixed in 10% buffered formalin solution, embedded in paraffin and cut in to 4 μm thick sections. After deparaffinization, the sections were stained with haematoxylin and eosin.

The activities of aspartate aminotransferase (AST), alanine aminotransferase (ALT) and creatine kinase (CK) were measured in the sera of chickens. The activity of AST and ALT was measured with DIALAB kits (Vienna, Austria) while CK activity was determined with CHRONO (Switzerland) reagents using on RA-1000 Technicon biochemical analyzer (New York, U.S.A.).

All morphometric data of the gastric lesions were statistically analyzed by the Mann Whitney U test between two independent groups. The enzyme activity data were statistically analyzed by Student's t-test. The level of significance was $P \leq 0.05$.

Results

Gross findings and morphometric analysis of gizzard lesions (Fig. 1)

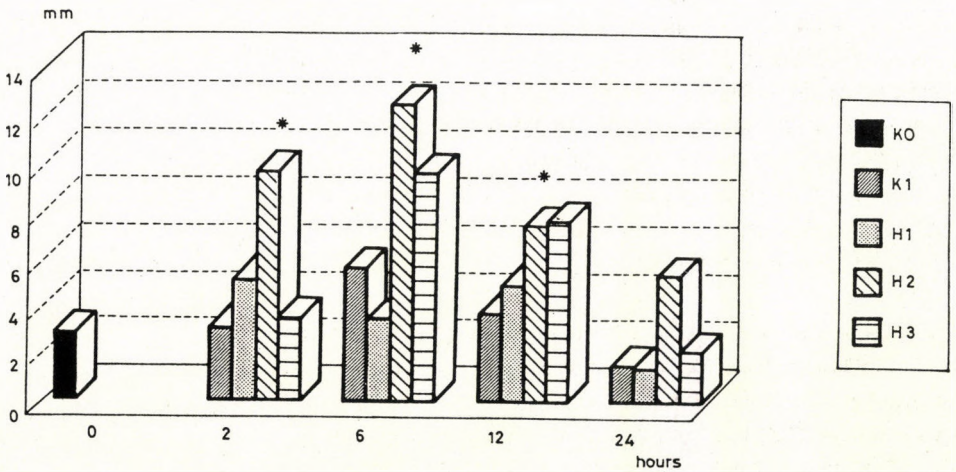


Fig. 1. Mean lengths (mm) of gizzard erosions in histamine-treated chickens.
K0: untreated healthy chicks; K1: 0.5 ml of saline per os; H1: histamine 5 mg/kg per os; H2: histamine 10 mg/kg per os; H3: histamine 15 mg/kg per os.
*$p < 0.05$ Mann-Whitney U test

In group K0, gizzards were intact in two cases, while in the remaining eight chicks there were mild lesions characterized by brownish discoloration at the glandular-gizzard junction. The average length of the lesions was 2.6 mm.

In group K1, birds sacrificed 2, 6, 12 and 24 h after the administration of saline had a mean lesion length of 3.05, 5.6, 3.7 and 1.5 mm, respectively. In four cases the gizzards were intact, in 5 cases the lesions were very mild and in 1 case discolorations with marked changes and article elevation at the glandular-gizzard junction was observed 2 h after administration. In the group of chickens sacrificed 6 h after saline administration, the gizzard was intact in only one case, in 4 cases mild superficial changes were observed, in 5 cases gizzard erosions with mild haemorrhages in to the gastric lumen but without melaena were seen. After 12 h the changes were much milder (4 intact, 4 with mild changes, 2 with erosions), while after 24 h the severity of lesions decreased (6 intact, 4 very mild changes).

In group H1, in chickens sactificed 2, 6, 12 and 24 h after the administration of histamine the mean total length of gizzard lesions was 5.1, 3.45, 4.85, and 1.4 mm, respectively. The gross changes were similar to those seen in group K1.

In group H2 the mean length of gizzard lesions was 9.7, 12.5, 7.4 and 5.3 mm in chickens sacrificed 2, 6, 12 and 24 h after histamine administration, respectively. Statistical significance was demonstrated between this group and group K1 in subgroups sacrificed 2, 6 and 12 h after histamine administration. All the chickens in this group (except those sacrificed after 24 h) had moderate to severe changes. In the group of birds sacrificed 24 h after histamine administration lesions were not present in three chickens, very mild lesions were seen in five chickens, and gizzard erosions and ulceration were found in two birds.

The gizzard lesions seen in group H3 were similar to those from group H1, i.e. predominantly mild changes were found. The mean length of gizzard lesions in this group was 3.5, 9.6, 7.6 and 2.1 mm in chickens sacrificed 2, 6, 12 and 24 h after histamine administration, respectively. Although the total length of lesions was quite increased as compared to group K1 especially in the subgroups of chicks euthanatized after 6 and 12 h, it did not reach the level of significance.

Histopathological findings

In group K0, the histopathological changes appeared in the form of discolorations with scattered areas of brown pigmentation.

In group K1 the commonest finding was the presence of a whitish and thinned cuticle with interspersed vacant spaces in chickens sacrificed after 2 h (Fig. 2). In chickens sacrificed after 6 and 12 h only discoloration was found. In birds sacrificed after 24 h the absence of boundaries between cuticle and glands was found, accompanied by discoloration.

In group H1, chickens sacrificed 2 h after histamine administration had a thin cuticle and an irregular focal thickening. In chickens sacrificed after 12 h nu-

merous vacant spaces in the cuticle were found, with scattered desquamation of cells and haemorrhages in the gizzard (Fig. 3).

Group H2 chickens sacrificed after 2 h showed a more severe thinning of cuticle with hyperproduction of the kaolin lining. In chickens sacrificed after 6 h histopathological changes were more severe, with hyperproduction of keratinous, villous perforated kaolin between atrophic glandular cells (Fig. 4). In birds sacrificed after 12 h histopathological changes were present as vacuolar hyperkeratosis of the cuticle with interspersed thinning. In chickens sacrificed after 24 h the cuticle was thinned and glandular atrophy with immature kaolin proliferation was observed.

The lesions seen in group H3 were very similar to those described in group H2.

Biochemical analysis of sera

The mean values of enzyme activities are shown in Figs 5, 6 and 7. The most consistent changes were found in the ALT values.



Fig. 2. Pale and vacuolated kaolin layer with atrophic and degenerated glands. Haematoxylin and eosin (H.-E.) × 10

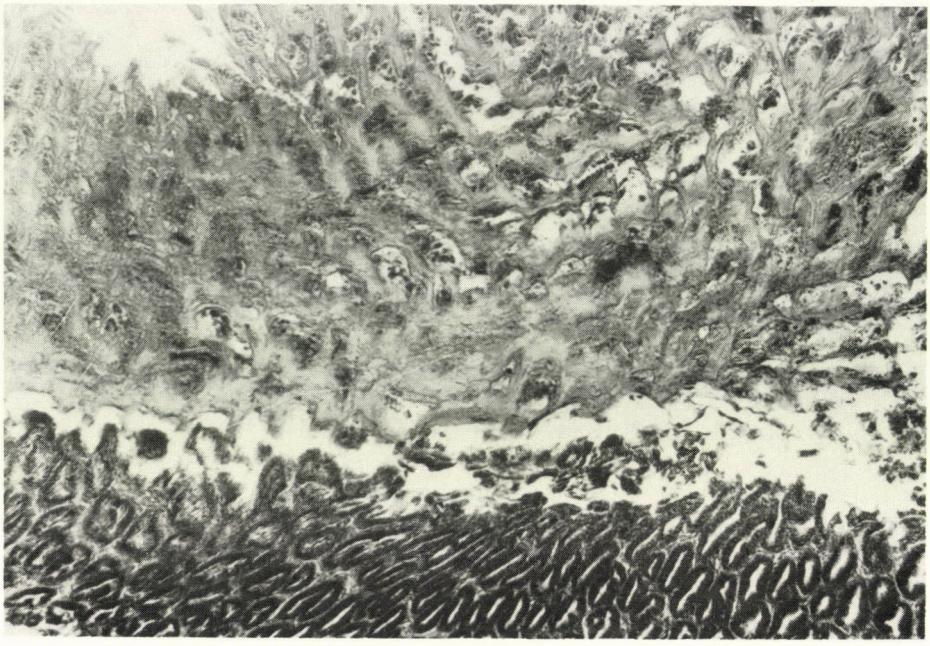


Fig. 3. Vacuolated cuticle with dispersed haemorrhages and accumulations of desquamated glandular cells. H.-E., × 10

Discussion

The administration of saline only (K1) did not cause statistically significant changes (gizzard lesions), i.e. there were no differences between this group and the healthy untreated control (K0). Mild lesions observed in group K0 were probably due to stress during transport on the first day of life. The findings for group K1 were different but not in a statistically significant degree. Similar results were obtained for groups H1 and H3 treated with 5 and 15 mg/kg b.m. histamine. Interesting results were obtained in group H2 (treated with 10 mg/kg b.m. histamine) where a statistically significant increase in the severity of gizzard lesions was seen, as compared with group K1 (groups of chicks treated with saline), in the subgroups sacrificed 2, 6 and 12 h after administration. The lesions were also very prominent in this group after 24 h, but there was no statistical significance, probably because the stadard deviation was high. These results clearly show histamine aggravates GE but only at the dose of 10 mg/kg b.m. Paré and Glavin (1986) cited studies in which various doses of histamine caused completely different effects in the same model of stress erosions. Our findings are consistent with the abovementioned results, i.e. the highest and lowest dose of histamine (5 and 15 mg/kg

b.m.) evoke milder erosions than the dose of 10 mg/kg b.m., although GE in chicks treated with the highest dose largely correspond to those provoked with a lower dose (10 mg), which can learly be seen from Fig. 1. Harry et al. (1975) and Harry and Tucker (1976) induced gizzard erosions in chickens with histamine administered at 4 mg/g of feed, while Itakura et al. (1982) were unable to reproduce the results of the former authors. None of the authors have investigated the dose- and time-dependent effect of histamine on gastric lesions and enzyme activities (AST, ALT and CK). We believe that because of the high biological variability between individuals, the administration of histamine via the feed is not a precise method for studying its effects.

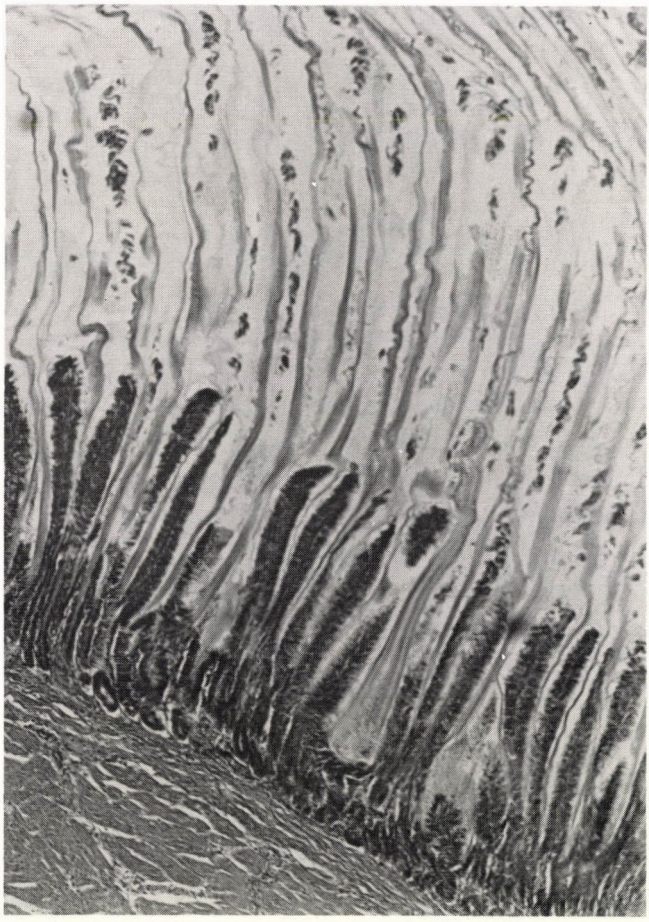


Fig. 4. Extended interglandular spaces with hyperproduction of villous material similar to kaolin. H.-E., × 10

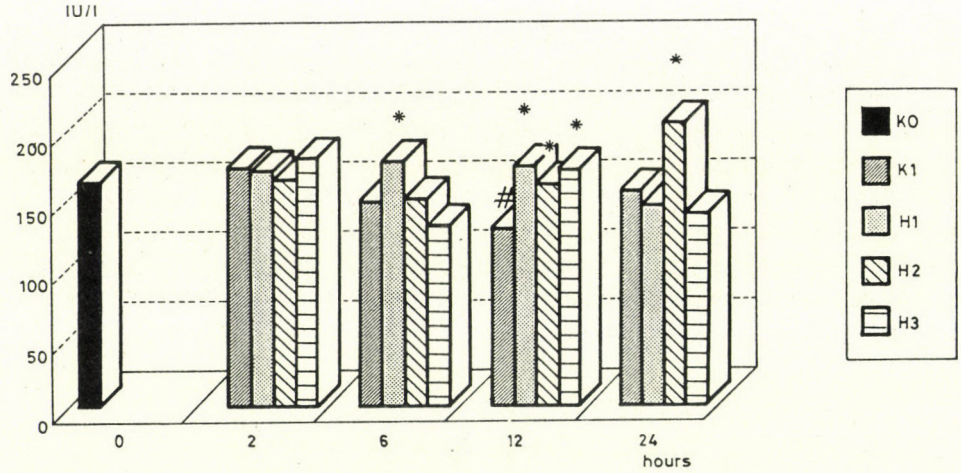


Fig. 5. Mean serum activities of aspartate aminotransferase (AST; IU/l) in the histamine-treated chicks. K0: untreated healthy chicks; K1: 0.5 ml of saline per os; H1: histamine 5 mg/kg per os; H2: histamine 10 mg/kg per os; H3: histamine 15 mg/kg per os. *$p < 0.05$ vs K1 at the same time; # $p < 0.05$ K1 vs K0 Student's t-test

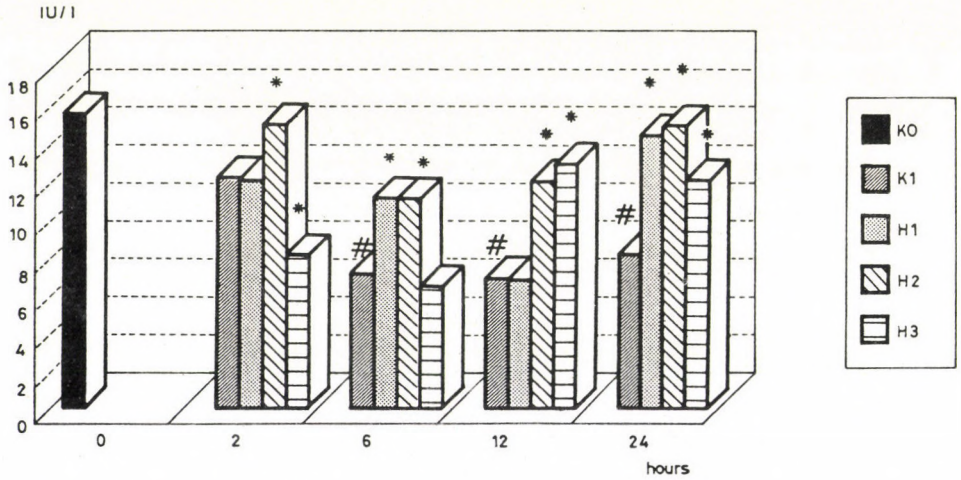


Fig. 6. Mean serum activities of alanine aminotransferase (ALT; IU/l) in the histamine-treated chicks. K0: untreated healthy chicks; K1: 0.5 ml of saline per os; H1: histamine 5 mg/kg per os; H2: histamine 10 mg/kg per os; H3: histamine 15 mg/kg per os. *$p < 0.05$ vs K1 at the same time; # $p < 0.05$ K1 vs K0 Student's t-test

The localization of lesions predominantly at the proventriculus-gizzard junction corresponds to the field cases of GE occurring spontaneously (Johnson and Piendo, 1971; Zorko, 1974; Kántás and Dobos-Kovács, 1990). Morphometri-

cal analysis of the gizzard erosions showed that the gizzard lining heals at a very fast rate: in all experimental and control groups of chickens the lesions almost completely disappear by 24 h after application. Szabó (1987) pointed out that the healing of gastric ulcers in mammals may occur by restitution, regeneration and reparation. Restitution is a very quick process involving migration of the mucosal epithelial cells towards the damage and lasts several hours, whilst regeneration includes proliferation of the epithelium and lasts longer than 1–2 days, depending on the duration of the cell division cycle. Reparation includes the proliferation of connective tissue (Baker and Van Dreumel, 1985). In our cases, the changes were mild even when bleeding was present, damages were superficial and healed by restitution or regeneration. The histopathological findings also suggest that histamine increases the production of the immature kaolin layer.

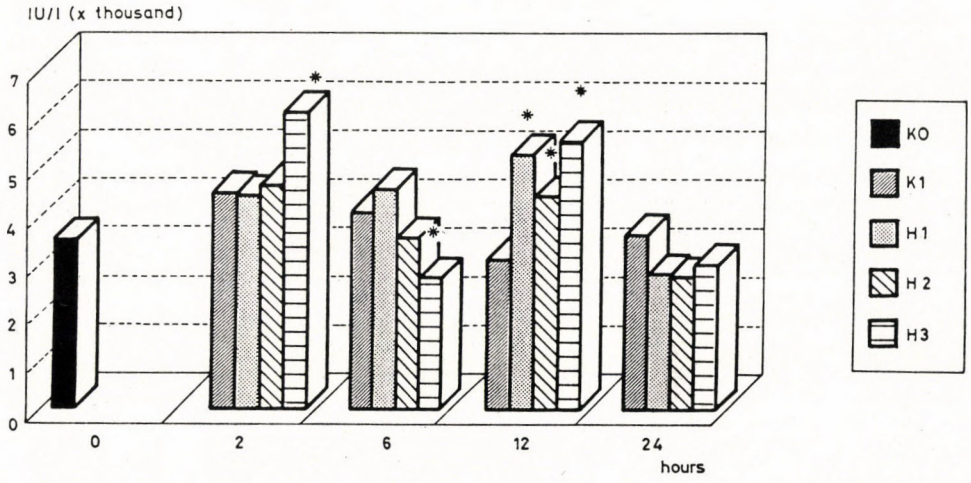


Fig. 7. Mean serum activities of creatine kinase (CK; IU/l) in the histamine-treated chicks. K0: untreated healthy chicks; K1: 0.5 ml of saline per os; H1: histamine 5 mg/kg per os; H2: histamine 10 mg/kg per os; H3: histamine 15 mg/kg per os. *$p < 0.05$ vs K1 at the same time, Student's t-test

Campbell and Coles (1986) stated that AST activity in birds depends on the species and it is the highest in the heart, muscles and liver of chickens and geese. Elevations higher than 230 IU/l are regarded as pathological. AST activity can be a valuable marker of milder tissue damages, and it may rise after the intramuscular application or a stress-related elevation of glucocorticoids. In our study, AST activity did not reach a pathological level, i.e. was not higher than 230 IU/l, but a significant increase was seen in all histamine-treated groups compared to group K1 in birds sacrificed 12 h after histamine application. In group K1, AST activity was significantly decreased compared with group K0 12 h after saline application,

which may probably be explained by diurnal variations. ALT activity significantly decreased in the control (K1) group compared with the healthy untreated control (K0) group 6, 12 and 24 h after administration. This decreased activity may also be the consequence of diurnal variations but physiological stabilization of the cell membrane and decreased enzyme release cannot be excluded either. However, in the treated groups increased activity was measured which was most consistent in group H2 where a significant increase was found at every interval compared to the control. This finding corresponds to data of the literature (Campbell and Coles, 1986) which, reported elevated activity of this enzyme in chickens and ducks with hepatic injury.

Mehner and Hartfiel (1983) reported that CK activity in one-day-old Leghorn chicks was between 1000 and 5000 IU/l, in 10 days old birds it decreased to 600–1400, while in birds older than 11 weeks it was 200–900 IU/l. Our results show that CK activity was not elevated in group K1 compared with group K0, while in the treated groups its variations were not consistent. It is important to note that elevation after 2 h was significantly above the maximum values described in the literature (6096 IU/l) in birds treated with the highest dose of histamine, which was also observed, although in a lesser degree, in chickens sacrificed after 12 h.

Although it is quite difficult to assess whether it is due to the direct influence of histamine on liver and other parenchymatous organs or induced GE, from these results we can conclude that, of the enzyme studied, the activity of ALT is the most sensitive and the most responsive to histamine administration.

References

Baker, I. K. and Van Dreumel, A. A. (1985): The alimentary system. In: Jubb, K. V. F., Kennedy, P. C. and Palmer, N. (eds) Pathology of Domestic Animals. 3rd edition, Vol. III, Academic Press. Orlando – San Diego. pp. 2–240.

Campbell, T. W. and Coles, E. H. (1986): Avian clinical pathology. In: Coles, E. H. (ed.) Veterinary Clinical Pathology. W. B. Saunders Company. Philadelphia, pp. 279–302.

Grabarević, Ž., Tišljar, M., Džaja, P., Artuković, B., Seiwerth, S. and Sikirić, P. (1993): Stress-induced gizzard erosion in chicks. J. Vet. Med. A. **40,** 265–270.

Harry, E. G., Tucker, J. F. and Laursen-Jones, A. P. (1975): The role of histamine and fish meal in the incidence of gizzard erosion and proventricular abnormalites in the fowl. Br. Poult. Sci. **16,** 69–78.

Harry, E. G. and Tucker, J. F. (1976): The effect of orally administered histamine on the weight gain and development of gizzard lesions in chicks. Vet. Rec. **99,** 206–207.

Hopkins, D. T., Armstrong, D. J., Struthers, B. J., Cover, M. S. and Rinehard, K. E. (1976): Factors influencing the incidence of gizzard erosion in chicks. Poultry Sci. **55,** 2046.

Horaguchi, M., Masumura, T., Horikawa, H. and Sugahara, M. (1980): Gizzard erosion and ulceration in broiler chicks. Effect of fish meal. Jpn. Poultry Sci. **17,** 351–357, Cited in: Vet. Bull. (1981) No. 5094.

Itakura, C., Hakotani, Y., Goto, M., Saito, T. and Ishii, K. (1981): Histopathology of gizzard erosion in young broiler chickens due to fish meal in the diets. Jpn. J. Vet. Sci. **43,** 677–687.

Itakura, C., Kazama, T. and Goto, M. (1982): Comparative pathology of gizzard lesions in broiler chicks fed meal histamine and copper. Avian Pathol. **11,** 487–502.

Janssen, W. M. (1971): The influence of feeding on gizzard erosion in broilers. Arch. Geflügelkde **35,** 137–141.

Johnson, D. C. and Piendo, D. (1971): Gizzard erosion and ulceration in Peru broilers. Avian Dis. **15,** 835–837.

Kántás, K. and Dobos-Kovács, M. (1990): Ulcerative gizzard disease in broiler chickens and laying hens (in Hungarian, with English abstract). Magyar Állatorvosok Lapja **45,** 93–97.

Kuba, K., Miyazaki, S. and Umemura, Y. (1983): Contents of free histidine and histamine in fish meals and in the model compounds and their toxicites to induce gizzard erosion. Natl Inst. Anim. Hlth Quart Japan **23,** 69–70. Cited in: Vet. Bull. (1984) No. 3657.

Kubena, L. F., Sadler, C. R., Haynes, R. L., Vardaman, T. H. and Deaton, J. W. (1976): Effect of fish and poultry byproduct meal on the small intestine and gizzard of broilers. Poultry Sci. **55,** 30–33.

Masumura, T., Horaguchi, H., Horikawa, H. and Sugahara, M. (1981): Gizzard erosion and ulceration in broilers. Toxic substances in fish meal. Jpn. Poultry Sci. **18,** 98–104. Cited in: Vet. Bull. (1981) No. 6975.

Mehner, A. and Hartfiel, W. (1983): Handbuch der Geflügelphysiologie. VEB Gustav Fischer Verlag, Jena.

Okazaki, T., Noguchi, T., Igarashi, K., Sakagami, Y., Seto, H., Mori, K., Naito, H., Masumura, T. and Sugahara, M. (1983): Gizzerosine, a new toxic substance in fish meal causes severe gizzard erosion in chicks. Agric. Biol. Chem. **47,** 2949–2952.

Paré, W. P. and Glavin, G. B. (1986): Restraint stress in biomedical research. A review. Neurosci. Biobehav. Rev. **10,** 339–370.

Rinehart, K. E., Cover, M. S., Hopkins, D. T. and Struthers, B. J. (1976): Effect of nutrient level ingredients and compounds on gizzard erosion in broilers. Poultry Sci. **55,** 2084.

Szabó, S. (1989): Critical and timely review of the concept of gastric cytoprotection. Acta Physiol. Hung. **73,** 115–127.

Umemura, Y., Miyazaki, S., Yamanaka, H., Ohya, T., Homma, S., Oka, M., Sato, S. and Nakahara, T. (1981): Properties of gizzard erosion-inducing substance in fish meal. Natl. Inst. Anim. Hlth Quart. Japan, **21,** 52–60. Cited in: Vet. Bull. (1981) No. 6971.

Umemura, Y. (1982): Studies on gizzard erosion-inducing substance in fish meal. JARQ **16,** 125–130. Cited in: Vet. Bull. (1983) No. 5999.

Zorko, N. (1974): Contribution to the aetiology of gizzard erosion in chicks. Zbornik peradarski dani Portorož. pp. 163–171.

Acta Veterinaria Hungarica 43 (4), pp. 443–451 (1995)

DIFFERENCES IN THE THYROXINE, TRIIODOTHYRONINE AND REVERSE TRIIODOTHYRONINE CONTENTS OF FETAL PIG TISSUES RELATIVE TO GESTATIONAL AGE

Ewa KRYSIN

Centre for Agrotechnology and Veterinary Sciences, Polish Academy of Sciences, Department of Developmental and Experimental Endocrinology, ul. Grunwaldzka 250, 60–166 Poznań, Poland

(Received July 3, 1995)

The concentrations of thyroxine (T_4), 3,5,3'-triiodothyronine (T_3) and 3,3',5'-triiodothyronine (reverse T_3) were measured in the liver, kidney and skeletal muscles of pig fetuses from day 32 to 113 of age. The iodothyronines were found to be present already between days 32 and 39, that is before the onset of fetal thyroid function. In the liver, kidneys and skeletal muscles T_4 reached maximal concentrations at midgestation (days 56 and 93). The T_3 level increased with gestational age in all tissues examined but, as opposed to T_4, its maximal value was reached shortly before birth. The rT_3 level was the highest between days 85 and 100 in the liver and kidney; however, in the skeletal muscles it did not change with gestation. The presented profiles of change of T_4 and rT_3 in the examined tissues did not correspond to the blood hormone pattern already observed in pig, whereas changes in tissue T_3 concentrations were similar to those in the serum.

Key words: Fetal pigs, thyroid hormones, liver, kidney, skeletal muscles

Relatively little information exists on the thyroid hormone (TH) concentrations in fetal tissues during gestation. Besides, the majority of available observations concern TH in fetal tissues of the rat. The level of iodothyronines (T_4 and T_3) was measured after homogenization of 10–12 days old rat embryo-trophoblasts or 13–21 days old embryos (Obregon et al., 1984; Escobar del Rey et al., 1986). In addition, it was determined in the fetal rat liver, brain, lung, heart and carcass on days 21 and 22 (Escobar del Rey et al., 1986; Morreale de Escobar et al., 1988; Alaez et al., 1992), and between days 17 and 22 (Ruiz de Ona et al., 1991). The rT_3 was described in measurable quantities in the fetal rat brain, carcass and liver on days 13 and 16 of gestation (Porterfield and Hendrich, 1992).

In humans, T_4 and T_3 concentrations in the brain, heart, kidney, liver, lung, skeletal muscles and skin were determined in 6 to 12 weeks old embryos, in 15 to 36 weeks old fetuses (Costa et al., 1991) and at midgestation (10–18 weeks) (Bernal and Pekonen, 1984).

0236–6290/95/$ 5.00 © 1995 *Akadémiai Kiadó, Budapest*

In the most recent work from this laboratory (Ślebodziński and Brzezińska-Ślebodzińska, 1994) it has been found that in the blood of prenatal pigs, serum T_4 and T_3 predominate while rT_3 is low, also in comparison with the maternal values. This blood hormone pattern is opposite to that found in preterm humans (Fisher et al., 1970; Fisher et al., 1973; Fisher, 1975), sheep (Fisher et al., 1972; Chopra et al., 1975), and rats (Harris et al., 1978; Calvo et al., 1990). In view of these species differences it was interesting to study the pattern of iodothyronine concentrations in pig fetuses.

The present paper is the first to show the T_4, T_3 and rT_3 contents of the liver, kidney and skeletal muscles of pig fetuses from day 32 of gestation until birth. The relative concentrations of the thyroid hormones in the fetal and maternal tissues during two phases, i.e. before and after the onset of porcine fetal thyroid function, are described.

Materials and methods

Animals. Pig fetuses (n = 80) from 11 litters obtained by Caesarian section from days 32 to 113 of gestation were used in the experiments. The liver, kidney and skeletal muscle were quickly dissected out and placed immediately in ice-cold 0.1 M Tris-HCl (pH 7.4) buffer.

Hormone extraction. Out of four methods most frequently used for TH measurement in tissues the one described by Chopra et al. (1982) proved to be the best in our hands (Krysin, 1990) and was utilized in this work. In short, tissue samples were homogenized in 2 vol (w/v) of ice-cold 0.1 M Tris-HCl buffer (pH 7.4) containing 2 mM PTU and the iodothyronines were extracted with 95% ethanol (4 ml of ethanol to 1 ml of homogenate). After storage for 24 h at –20 °C the extract was centrifuged at 2,500 × g for 30 min at 4 °C. The supernatant was separated and the pellet was extracted again with 2 ml of ethanol and kept overnight at –20 °C. After centrifugation both supernatant fractions were pooled and stored at –20 °C until the analysis.

Radioimmunoassay. The iodothyronines were measured in the ethanol extracts by RIA (Ślebodziński et al., 1980) and expressed as ng of hormone per g wet tissue. ^{125}I-labelled T_4, T_3 and rT_3 of specific activity over 1200 μCi/μg were obtained from the Institute of Isotopes, Budapest. The cross-reactivity of the antibodies was negligible (Ślebodziński et al., 1982). The RIA procedure detected 2.2 nmol T_4/l and 0.11 nmol T_3 or rT_3 /l in 0.025–0.1 ml samples of ethanol extracts. Each sample was determined in triplicate.

Statistical analysis. Data were given as means ± SEM. Student's *t*-test and analysis of variance were applied. Significance was defined as $P < 0.05$.

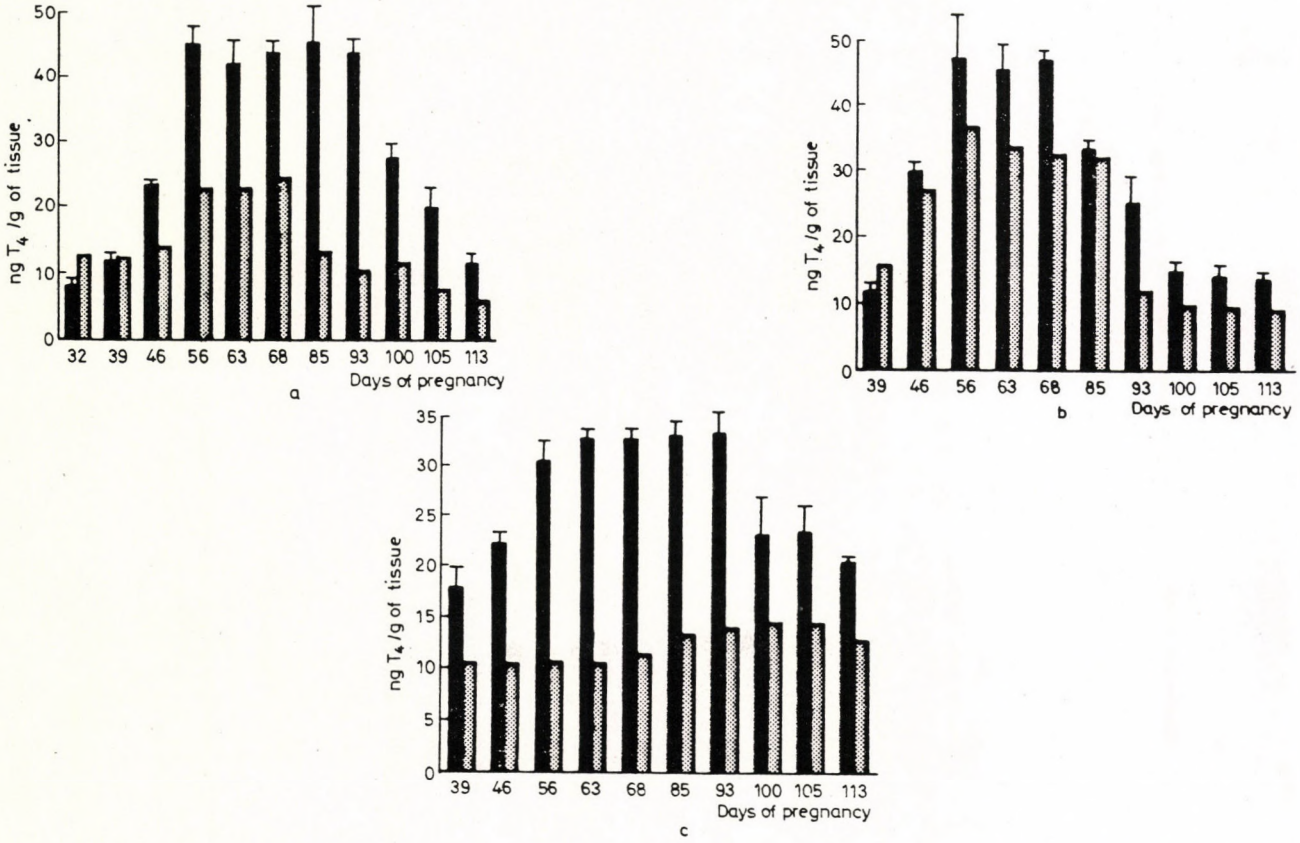


Fig. 1. Changes in thyroxine (T_4) concentrations in fetal (solid bars) and maternal (shaded bars) liver (a), kidney (b) and skeletal muscles (c). Values are means ± SEM; n = 5–9

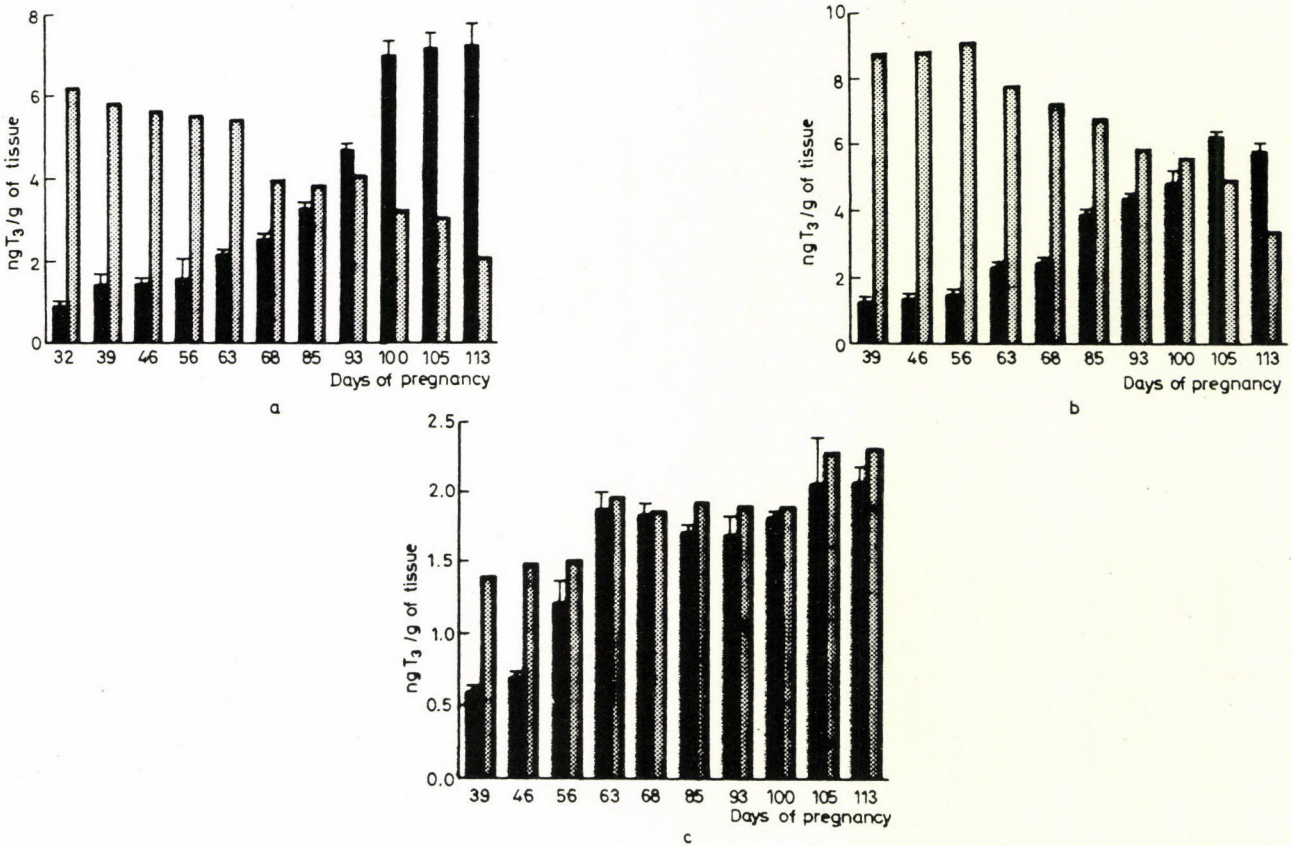


Fig. 2. Changes in triiodothyronine (T_3) concentrations in fetal (solid bars) and maternal (shaded bars) liver (a), kidney (b) and skeletal muscles (c). Values are means ± SEM; n = 5–9

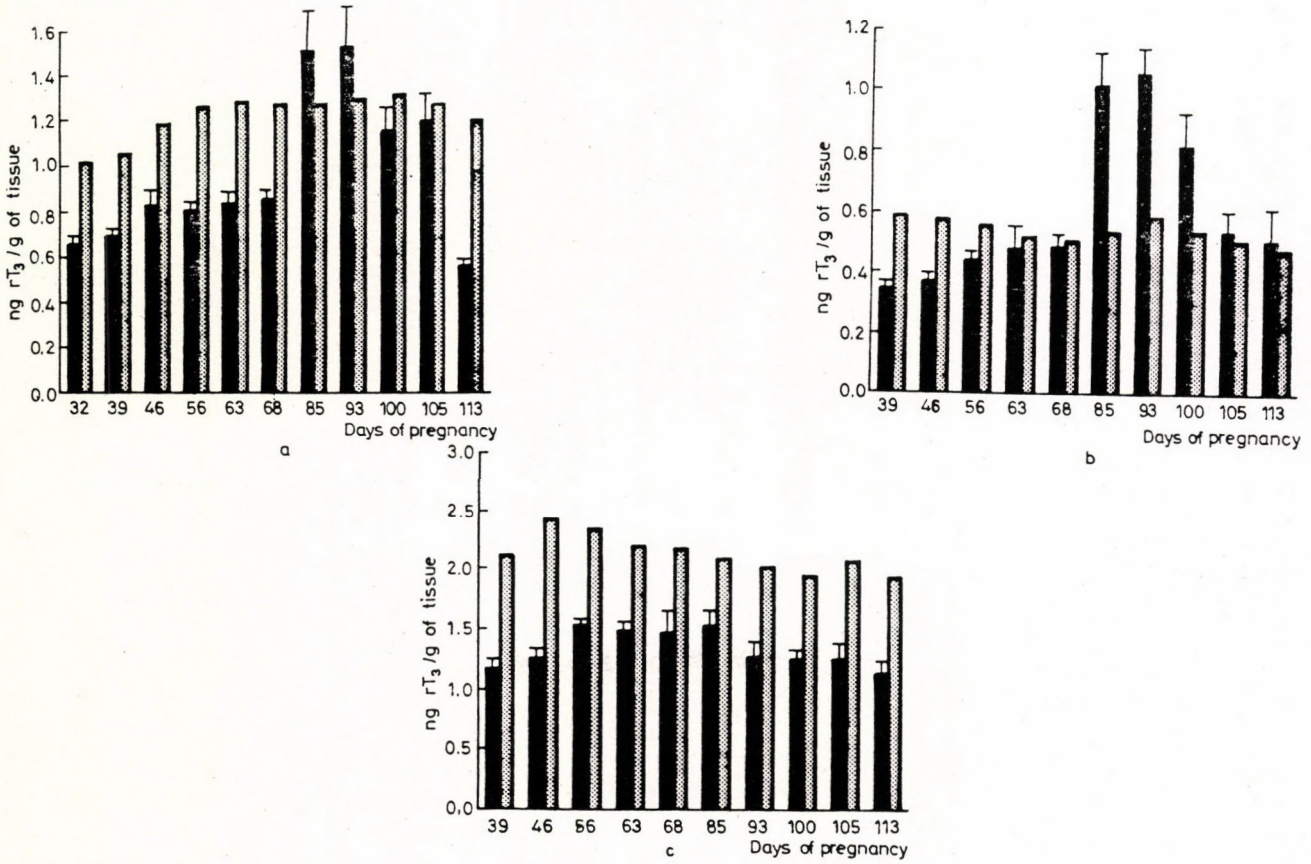


Fig. 3. Changes in reverse triiodothyronine (rT_3) concentrations in fetal (solid bars) and maternal (shaded bars) liver (a), kidney (b) and skeletal muscles (c). Values are means ± SEM; n = 5–9

Results

The presence of measurable levels of thyroxine and both triiodothyronines in the liver, kidney and skeletal muscles was observed from 32–39 days of fetal life (the beginning of our studies).

The concentrations of T_4 in the fetal liver (Fig. 1a) increased 2- to 3-fold between days 56 and 93 of gestation (44.2 ± 0.61 ng/g, n = 29) and markedly decreased shortly before birth (11.8 ± 0.96 ng/g, n = 6). The T_4 content in the kidney (Fig. 1b) was the highest between days 56 and 68 of gestation (46.8 ± 0.60 ng/g, n = 20) and then gradually decreased to 14.9 ± 1.32 ng/g, n = 5 on day 100. On day 68 of gestation the level of T_4 (47.2 ± 1.33 ng/g, n = 6) in fetal kidney (Fig. 1b) was about four times higher than on day 113 (13.8 ± 0.57 ng/g, n = 8). In the fetal skeletal muscles (Fig. 1c) the T_4 concentrations increased to the peak values between days 56 and 93 (32.6 ± 0.51 ng/g, n = 29), but during the last two weeks of gestation the T_4 level was significantly lower ($P < 0.01$). From day 39 of gestation in skeletal muscles, and from day 46 in the liver and kidney until term, the fetal tissues showed higher T_4 concentrations than the maternal tissues (Fig. 1a, b, c). Contrary to the liver and the kidney, in the maternal skeletal muscles the T_4 level was relatively stable (12.4 ± 0.55 ng/g, n = 10) throughout pregnancy.

The T_3 levels in the fetal liver (Fig. 2a) and kidney (Fig. 2b) demonstrated differences ($P < 0.01$) between the first (32–68 days) and the second (93–113 days) part of gestation when they increased about 7-fold. The T_3 concentrations of fetal tissues rose with the gestational age from 0.9 ± 0.08 to 7.3 ± 0.49 ng/g (n = 5–6) in the liver and from 1.3 ± 0.15 to 6.2 ± 0.14 ng/g (n = 6–7) in the kidney. In the fetal skeletal muscle (Fig. 2c) the level of T_3 increased three-fold by day 63, but between days 63 and 113 it did not change (1.9 ± 0.06 ng/g, n = 40). The T_3 content in the fetal liver and kidney during the last two weeks of gestation was significantly higher ($P < 0.01$) in relation to that in maternal tissue, contrary to fetal skeletal muscles where it was lower ($P < 0.01$) than in the mothers throughout pregnancy. In both maternal and fetal skeletal muscles, the T_3 concentrations (Fig. 2c) were generally lower than in the liver and kidney (Fig. 2a, b).

The rT_3 concentrations in the fetal liver (Fig. 3a) and kidney (Fig. 3b) showed insignificant variations between days 32 and 68 of gestation (liver 0.8 ± 0.02 ng/g, n = 31; kidney 0.4 ± 0.03 ng/g, n = 28) but rose rapidly ($P < 0.01$) on days 85 and 93 reaching a maximal value (1.5 ± 0.18 ng/g, n = 5) in the liver and (1.1 ± 0.09 ng/g, n = 6) in the kidney. Near term the fetal rT_3 level markedly decreased to 0.6 ± 0.02 ng/g, $P < 0.001$ (n = 6) in the liver and to 0.5 ± 0.10 ng/g, $P < 0.01$ (n = 8) in the kidney. In the fetal skeletal muscles the rT_3 concentration was unchanged (1.3 ± 0.05 ng/g, n = 63) throughout pregnancy. The rT_3 concentrations of the maternal tissues were stable during pregnancy and the mean hor-

mone levels were 1.2 ± 0.03 ng/g (n = 11) in the liver; 0.5 ± 0.01 ng/g (n = 10) in the kidney, and 2.1 ± 0.05 ng/g (n = 10) in the skeletal muscles. Compared with the maternal tissues the fetal rT_3 concentrations were lower in the liver and skeletal muscles or similar in the kidney. Only between days 85 and 100 were the rT_3 levels significantly higher in the fetal liver ($P < 0.01$) and kidney ($P < 0.001$) than in the corresponding maternal.

Discussion

There is a lack of information on the thyroid hormone concentrations of fetal pig tissues. The present study is the first in which the patterns of developmental changes in the thyroid hormone contents (T_4, T_3 and rT_3) in the liver, kidney and skeletal muscles were described. The fetal period studied comprised the time before the onset of fetal thyroid function up to the day of birth. The observed increase in the fetal tissues' iodothyronines agrees with the beginning of the pig thyroid secretory activity; about day 52 of gestation (Rankin, 1941).

It has been found that T_4, T_3 and rT_3 are measurable by RIA in the liver from day 32, in the kidney and skeletal muscles from day 39 of gestation, that is before the onset of fetal thyroid activity. This finding indicates that thyroid hormones of maternal origin are present and might have a role in early embryonic development. Besides, it shows that there is a placental transfer of iodothyronines before the fetal thyroid becomes functional. This result is consistent with some previous reports in rats (Sweney and Shapiro, 1975; Woods et al., 1984; Morreale de Escobar et al., 1985) showing the passage of iodothyronines from the mother to the fetus during the first two weeks of pregnancy. However, the rat is not a very suitable species to compare with the pig because its fetal thyroid activity starts shortly before birth (Morreale de Escobar et al., 1985; Nataf and Sfez, 1961), whereas the pig thyroid is active already at midgestation.

The T_4 level in the fetal pig tissues (liver and kidney) was shown to be significantly higher than in the maternal tissues from day 56, and decreased near term, which is contrary to the data presented for the liver of fetal rats (Ruiz de Ona et al., 1991). On the other hand, the T_3 levels in the fetal pig liver and kidney rise with age parallel to T_3 concentrations in fetal blood (Ślebodziński and Brzezińska-Ślebodzińska, 1994). An increased T_3 concentration in fetal liver and lungs from days 17 to 22 was noticed in rat (Ruiz de Ona et al., 1991) and between weeks 15 and 36 in some tissues of human fetuses (Costa et al., 1991). The marked increase in T_3 concentrations in the pig fetal liver (from days 100–105 of gestation), accompanied by the decrease of T_4 levels, might indicate enhanced generation of T_3 from T_4 near term.

The rT_3 level was much lower than T_4 (about 40 times) and than T_3 (about 7 times) in the pig fetal liver. In the rat the liver rT_3 concentrations in 16 days old

fetuses are 6 and 5 times lower when compared with that of T_4 (Porterfield and Hendrich, 1992).

Compared with the maternal tissue, T_4 concentrations in fetal skeletal muscles were 3 times higher, T_3 concentrations were similar and the rT_3 level was about 25% lower.

The T_4 levels in fetal skeletal muscles were 27% and 31% lower than that in fetal liver and kidney, respectively. The T_3 concentrations in the fetal skeletal muscles were 3.5 times lower than in fetal liver and kidney. Considering the big mass of skeletal muscles, this tissue seems to contribute significantly to the iodothyronines pool in the fetus.

The present data show that T_4, T_3 and rT_3 are present in the pig fetal tissues before the onset of fetal thyroid function. Their levels increase significantly from about the beginning of the fetal thyroid secretion, reach a peak at the middle of gestation (T_4, rT_3) or near term (T_3). The obtained data support the view that, in the pig, the placenta is permeable to thyroid hormones before the onset of fetal thyroid activity, and that during this period the fetal tissues are under the influence of the maternal thyroid hormones. It is interesting to note that the presented profiles of change of T_4 and rT_3 in the examined tissues did not correspond to the blood hormone pattern observed in pigs (Ślebodziński and Brzezińska-Ślebodzińska, 1994) whereas the changes in the T_3 concentrations of tissues were similar to those in serum.

Acknowledgements

I wish to thank Miss M. Cholaszczyńska for T_4 determination and Mrs. K. Kowalska, M. Sci. for her help with statistics and the preparation of figures.

References

Alaez, C., Calvo, R., Obregon, M. J. and Pascual-Leone, A. M. (1992): Thyroid hormones and 5'-deiodinase activity in neonatal undernourished rats. Endocrinology **130,** 773–779.

Bernal, J. and Pekonen, F. (1984): Ontogenesis of the nuclear 3,5,3'-triiodothyronine receptor in the human fetal brain. Endocrinology **114,** 677–679.

Calvo, R., Obregon, M. J., Ruiz de Ona, C., Escobar del Rey, F. and Morreale de Escobar, G. (1990): Congenital hypothyroidism, as studied in rats. J. Clin. Invest. **86,** 889–899.

Chopra, I. J., Sack, J. and Fisher, D. A. (1975): 3,3',5'-triiodothyronine (reverse T_3) and 3,3',5-triiodothyronine (T_3) in fetal and adult sheep: studies of metabolic clearance rates, production rates, serum binding, and thyroidal content relative to thyroxine. Endocrinology **97,** 1080–1088.

Chopra, I. J., Chua Teco, G. N., Eisenberg, J. B., Wiersinga, W. M. and Solomon, D. H. (1982): Structure–activity relationship of inhibition of hepatic monodeiodination of thyroxine to 3,5,3'-triiodothyronine by thiouracil and related compounds. Endocrinology **110,** 163–168.

Costa, A., Arisio, R., Benedetto, C., Bertino, E., Fabris, C., Giraudi, G., Marozio, L., Maula, V., Pagliano, M. and Testori, O. (1991): Thyroid hormones in tissues from human embryos and fetuses. J. Endocrinol. Invest. **14,** 559–568.

Escobar del Rey, F., Pastor, R., Mallol, J. and Morreale de Escobar, G. (1986): Effects of maternal iodine deficiency on the L-thyroxine and 3,5,3'-triiodo-L-thyronine contents of rat embryonic tissues before and after onset of fetal thyroid function. Endocrinology **118,** 1259–1265.

Fisher, D. A., Hobel, C. J., Garza, R. and Pierce, C. A. (1970): Thyroid function in the preterm fetus. Pediatrics **46,** 208–216.

Fisher, D. A., Dussault, J. H., Erenberg, A. and Lam, R. W. (1972): Thyroxine and triiodothyronine metabolism in maternal and fetal sheep. Pediat. Res. **6,** 894–899.

Fisher, D. A., Dussault, J. H., Hobel, C. J. and Lam, R. (1973): Serum and thyroid gland triiodothyronine in the human fetus. J. Clin. Endocrinol. Metab. **36,** 397–400.

Fisher, D. A. (1975): Reverse tri-iodothyronine and fetal thyroid status. New Engl. J. Med. **293,** 770–771.

Harris, A. R. C., Fang, S. L., Prosky, J., Braverman, L. E. and Vagenakis, A. G. (1978): Decreased outer ring monodeiodination of thyroxine and reverse triiodothyronine in the fetal and neonatal rat. Endocrinology **103,** 2216–2222.

Krysin, E. (1990): Comparison of different methods for iodothyronines extraction from animal tissues. Polskie Archiwum Weterynaryjne **30,** 39–48.

Morreale de Escobar, G., Pastor, R., Obregon, M. J. and Escobar del Rey, F. (1985): Effcets of maternal hypothyroidism on the weight and thyroid hormone content of rat embryonic tissues, before and after onset of fetal thyroid function. Endocrinology **117,** 1890–1900.

Morreale de Escobar, G., Obregon, M. J., Ruiz de Ona, C. and Escobar del Rey, F. (1988): Transfer of thyroxine from the mother to the rat fetus near term: effects on brain 3,5,3'-triiodothyronine deficiency. Endocrinology **122,** 1521–1531.

Nataf, B. and Sfez, M. (1961): Debut de fonctionnement de la thyroide foetale de rat. C. R. Soc. Biol. (Paris) **156,** 1235–1238.

Obregon, M. J., Mallol, R., Pastor, G., Morreale de Escobar, G. and Escobar del Rey, F. (1984): L-Thyroxine and 3,5,3'-triiodo-L-thyronine in rat embryos before onset of fetal thyroid function. Endocrinology **114,** 305–307.

Porterfield, S. P. and Hendrich, C. E. (1992): Tissue iodothyronine levels in fetuses of control and hypothyroid rats at 13 and 16 days gestation. Endocrinology **131,** 195–200.

Rankin, R. M. (1941): Changes in the content of iodine compounds and in histological structure of the thyroid gland of the pig during fetal life. The Anatomical Record **80,** 123.

Ruiz de Ona, C., Morreale de Escobar, G., Calvo, R., Escobar del Rey, F. and Obregon, M. J. (1991): Thyroid hormones and 5'-deiodinase in the rat fetus late in gestation: effects of maternal hypothyroidism. Endocrinology **128,** 422–432.

Sweney, L. R. and Shapiro, B. L. (1975): Thyroxine and palatal development in the rat. Dev. Biol. **42,** 19.

Ślebodziński, A. B., Nowak, G. and Zamysłowska, H. (1980): A simple unifying procedure for radioimmunoassay of thyroxine, triiodothyronine and reverse triiodothyronine in unextracted serum. Endokrynologia Polska **31,** 537–548.

Ślebodziński, A. B., Brzezińska-Ślebodzińska, E. and Drews, R. (1982): Reciprocal changes in serum 3,3',5'-triiodothyronine concentration and the peripheral thyroxine inner ring monodeiodination during food restriction in the young pig. J. Endocr. **95,** 349–355.

Ślebodziński, A. B. and Brzezińska-Ślebodzińska, E. (1994): The appearance and activity of tissue thyroxine 5'- and 5-monodeiodinase during ontogenesis in the fetal pig. Journal of Endocrinology **141,** 243–249.

Woods, R. J., Sinha, A. and Ekins, R. (1984): Uptake and metabolism of thyroid hormones by the rat fetus in early pregnancy. Clin. Sci. **67,** 359–363.

Acta Veterinaria Hungarica 43 (4), pp. 453–461 (1995)

SEASONAL VARIATION IN THE CONCENTRATION OF VITAMINS A AND E IN THE BLOOD PLASMA OF FAT-TAILED SHEEP

A. ASADIAN[1,2], S. A. MIRHADI[2] and M. MÉZES[1]*

[1]Department of Nutrition, University of Agricultural Sciences, H–2103 Gödöllő, Hungary; [2]Animal Husbandry Research Institute, 31585 Karaj, Iran

(Received May 15, 1995)

Six non-pregnant ewes and 6 rams (age: 3–4 years) of an Iranian fat-tailed sheep breed (Shal) were used. Blood samples were collected monthly for 12 months, and the concentrations of retinol and α-tocopherol were determined by high-performance liquid chromatography (HPLC). A limited ration of standard composition was fed throughout the year. The ration was supplemented with 25–32 IU α-tocopherol/animal/day. Vitamin A concentrations in the blood plasma of ewes were lower in the spring (442 ± 9 μg/L) and summer (452 ± 7 μg/L) and higher in the autumn (467 ± 5 μg/L). In the rams, the plasma concentration of vitamin A was the lowest in the spring (436 ± 6 μg/L) and the highest in the summer (471 ± 5 μg/L). A seasonal comparison did not show statistical differences between consecutive seasons for the ewes ($P > 0.05$). The differences were significant between winter and spring ($P < 0.05$) and spring and summer ($P < 0.001$) for the rams. The blood plasma concentration of vitamin E was 1.21 ± 0.05, 1.04 ± 0.05, 1.24 ± 0.05 and 1.24 ± 0.08 mg/L in spring, summer, autumn and winter, respectively, for the ewes and 1.24 ± 0.05, 1.11 ± 0.06, 1.09 ± 0.04 and 1.38 ± 0.07 mg/L in spring, summer, autumn and winter, respectively, for the rams. The values were significantly different between spring and summer ($P < 0.05$) and summer and autumn ($P < 0.01$) for the ewes. In rams, the values obtained in autumn also showed a significant difference ($P < 0.001$) from those found in winter. Differences between values found in other seasons were not significant ($P > 0.05$). The retinol and α-tocopherol concentrations of the blood plasma were highly similar in the two sexes. The concentrations of retinol in the summer ($P < 0.01$) and α-tocopherol in the autumn ($P < 0.05$) showed significant differences between the two sexes. The results show that the concentrations of vitamins A and E are relatively constant in the blood plasma of fat-tailed sheep kept on a standardised feed. However, some differences due to the influence of season and sex were observed.

Key words: Fat-tailed sheep, vitamin A, vitamin E, blood plasma, seasonal variation

* to whom reprint requests should be addressed

0236–6290/95/$ 5.00 © 1995 *Akadémiai Kiadó, Budapest*

The level of vitamins A and E in the blood plasma is affected by several factors. Wooton (1962) reviewed the literature dealing with various sources of normal variations in blood constituents. In addition to specific individual variation, other factors such as age and sex, time of the day, season of the year, diet, geographical location, pasture and activity can also modify blood composition. Seasonal variation of vitamins A and E in blood may also differ in individual genotypes of the same species, e.g. sheep (Bedő et al., 1992), and different species of ruminants, e.g. cattle, sheep and goats, may have dissimilar variation patterns (Hidiroglou and Williams, 1986; Glover et al., 1976; Molokwu, 1978). The seasonal variation in the vitamin content of the blood follows dissimilar patterns in different species. In ewes, the blood contained higher levels of vitamins A and E in April and May, followed by a significant decrease in summer and autumn (Bedő et al., 1992). Glover et al. (1976) reported that in wethers minimal plasma vitamin A concentrations occurred in the summer, with vitamin A peaks in winter and a binding protein (holo-RBP) peak in September. Hidiroglou (1983) indicated that the season was not a significant source of variation for vitamin A and no relationship with temperature was apparent. At the same time, the season was found to be a significant ($P < 0.01$) source of variation for vitamin E in ovine blood. Molokwu (1978) reported that there was little or no seasonal variation of vitamin A in caprine blood and that the vitamin A level varied slightly between seasons. The plasma concentrations of vitamin E in cattle showed a marked seasonal pattern and the concentrations increased from January to a peak in July with a subsequent decrease in autumn (Hidiroglou and Williams, 1986).

The aim of this study was to measure the effects of season on the blood plasma level of vitamins A and E in a previously not investigated traditional fat-tailed sheep genotype given standardized feeding.

Materials and methods

Animals

Six non-pregnant ewes (age: 3–4 years, body mass: 65–70 kg) and six mature rams (age: 3–4 years, body mass: 65–75 kg) were used. The animals originated from a flock of fat-tailed sheep of the Shal breed (an Iranian breed). Before and during the experiment, the ewes were fed a ration of 1.65 kg/animal/day, consisting of poor-quality chopped alfalfa hay (66.7%), wheat straw (21.2%) and wheat bran (12.1%). The rams were fed a ration of 1.85 kg/animal/day, consisting of poor-quality chopped alfalfa hay (70.3%), wheat straw (18.9%) and wheat bran (10.8%). Feed samples were taken monthly to determine the α-tocopherol and β-carotene content.

The animals had free access to salt and water. The wheat bran was supplemented weekly with dl-α-tocopheryl acetate (Rovimix E-50 Adsorbate, F. Hoffmann-La Roche Ltd., Basle) to provide 25–32 IU α-tocopherol/animal.

Ewes and rams were housed separately inside an open shelter. Blood samples were obtained monthly from the jugular vein by venipuncture. Blood plasma was separated within 2 h and stored in airtight polypropylene tubes at –20 °C until used for the determination of retinol and α-tocopherol concentrations.

Analytical methods

The concentration of vitamins A and E in the blood plasma of animals was determined by high-performance liquid chromatography (HPLC) with an ultraviolet (UV) detector. For each sample parallel determinations were carried out.

Plasma (1 ml) protein precipitation was done with absolute ethanol (1 ml, containing 0.125% butyl-hydroxy-toluene, BHT) and extraction was done with n-hexane twice (5 ml, containing 0.025% BHT) by the extraction method of Biesalski et al. (1986) with some minor modifications aimed at improving the rate of recovery and by antioxidant application of as described by Chow and Omaye (1983). The extract was dried under nitrogen stream and the residue was dissolved in 0.5 ml methanol. An amount of 50 µl was injected into a Varian 5000 HPLC instrument equipped with reverse-phase column (Nucleosil C18, 250 × 4 mm) of 5 µm particle size (Bio-separation Technologies Co. Ltd., Budapest). The solvent was vortexed and sonicated for 1 min, followed by 15 min of centrifugation at 8,000 *g*. All solvents were analytical (absolute ethanol), extra pure (n-hexane) or HPLC grade (methanol) quality.

A common wavelength of 292 nm was used for the determination of retinol and α-tocopherol content. The eluent was methanol: water (97:3) with a flow rate of 1.5 ml/min. Peak areas were automatically recorded by means of a Varian integrator model 4290.

Retinol and dl-α-tocopherol standards were used for peak identification (Fluka Chemia-Bio-Chemika, Buchs). Identification and quantitation of the vitamins were accomplished by the comparison of retention time and peak area.

The α-tocopherol level of the feed was measured by the method of McMurray and Blanchflower (1979). Determination of β-carotene in the feed was done spectrophotometrically (Spectronic 20, Bausch and Lomb, Rochester) by the method of A.O.A.C. (1990).

Mathematical analysis

Analysis of variance (ANOVA) of the data was used for the estimation of the effect of season and sex on the vitamin A and E content of the blood plasma using a completely randomized design.

Results

Vitamin A concentration in the blood plasma was the lowest in the spring and summer for the ewes and in the spring for the rams. In the ewes, lower values were found in the winter and summer than in the autumn in ewes. However, the differences were not statistically significant ($P > 0.05$). In rams, the concentration of vitamin A in the blood plasma was lower in the spring than in winter ($P < 0.01$) and in summer ($P < 0.001$).

In ewes, the concentration of vitamin E in the blood plasma was the lowest in summer. The differences were statistically significant as compared to values found in the spring ($P < 0.05$) and autumn ($P < 0.01$). In rams, the lowest values were found in the summer and autumn. The differences between autumn and winter were statistically significant ($P < 0.001$). Higher values were observed in winter and spring (Table 1).

Table 1

Mean values of vitamins A and E in the blood plasma of sheep

Sex	Season	Vitamin A µg/L	SEM	CV%	Vitamin E mg/L	SEM	CV%
Ewes (n = 6)	spring	442	9.0	9.0	1.21	0.05	18.2
	summer	452	7.0	7.0	1.04	0.05	20.3
	autumn	467	5.0	4.7	1.24	0.05	18.5
	winter	458	7.0	6.8	1.24	0.08	25.8
Rams (n = 6)	spring	436	6.0	6.3	1.24	0.05	16.9
	summer	471	5.0	4.7	1.11	0.06	21.6
	autumn	460	4.0	3.7	1.09	0.04	14.7
	winter	461	7.0	6.7	1.38	0.07	20.3

SEM: standard error of mean; CV%: coefficient of variation

Sex-related differences within the seasons were observed for vitamin A in the spring ($P < 0.05$) and for vitamin E in the autumn ($P < 0.05$). The results are presented in Tables 1–5.

Discussion

The results indicate that the plasma concentrations of A and E did not remain on a steady level throughout the year in sheep fed a standardized diet.

Vitamin A concentration of the blood plasma of ewes showed little variation by season. The results are consistent with the previous findings of Hidiroglou

(1983) that time was not a significant source of variation for vitamin A level in the blood plasma of sheep. Molokwu (1978) also reported that there was little or no seasonal variation in the vitamin A content of caprine blood. However, in this study higher values were observed in the autumn and early winter period (Table 4). The differences were statistically not significant between the seasons. Moderately higher values of retinol were observed in autumn and early winter, which coincided with the time of sexual activity of ewes. Retinol is well known as a substance essential for reproduction (Thompson, 1969), and ewes are sexually active in autumn and winter but less so in late winter and spring. Glover et al. (1976) indicated that the concentration of holo-retinol binding protein (holo-RBP) remained high throughout the autumn and then decreased in December to a value similar to that found in January and February. This supports the above-mentioned hypothesis about the effect of sexual functions on the metabolism and/or transport of vitamin A.

Table 2

Effect of season on the level of vitamins A and E in the blood plasma of sheep (seasonal differences)

Sex	Seasonal comparison	Vitamin A μg/L	Vitamin E mg/L
Ewes (n = 6)	spring-summer	NS	*
	spring-winter	NS	NS
	summer-autumn	NS	**
	autumn-winter	NS	NS
Rams (n = 6)	spring-summer	***	NS
	spring-winter	**	NS
	summer-autumn	NS	NS
	autumn-winter	NS	***

NS: non-significant; *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$

Vitamin A concentration in the blood plasma of rams showed the same pattern as was found in ewes but the level of vitamin A started to increase before autumn (from July and August). This means that the metabolism and transport of vitamin A increased before the onset of the breeding season. This effect may be due to the different sexual endocrine changes in rams or to the dissimilar temperature sensitivity of rams and ewes.

In general, it can be concluded that vitamin A concentration of the blood plasma tended to show moderate changes, and sheep may use their vitamin A res-

ervoirs for maintaining a constant level in the circulation. However, some physiological requirement or excess intake may change the values to some extent.

Glover et al. (1976) reported that a major fluctuation in holo-RBP concentration occurred in both sexes of sheep, and if followed the same pattern as vitamin A concentration of the blood. The results of this were consistent with their findings. The lowest values for both sexes were found in the spring, but a significant difference in vitamin A was observed only in summer ($P < 0.05$).

The concentration of vitamin E (α-tocopherol) was generally lower in the summer in both sexes and followed more or less the same pattern throughout the year. A statistically significant difference between the sexes ($P < 0.05$) was found only in autumn, when the lower values were observed in rams (Table 3).

Table 3

Effect of sex on the level of vitamins A and E in the blood plasma of sheep (sex comparison; n = 6/sex)

Seasonal comparison	Vitamin A μg/100 ml	Vitamin E μg/ml
Spring	NS	NS
Summer	**	NS
Autumn	NS	*
Winter	NS	NS

NS: non-significant; *: $P < 0.05$; **: $P < 0.01$

Vitamin E concentration of the blood plasma of ewes showed a significant decrease ($P < 0.05$) in summer as compared to spring and a significant increase in autumn as compared to summer ($P < 0.01$). The rams showed a highly significant increase from autumn to winter ($P < 0.001$).

Both sexes showed higher values of vitamin E in colder seasons. The higher values can be attributed to the increased metabolic demand followed by a higher absorption in the colder seasons.

Vitamin E concentration tended to increase from August up to December in the blood plasma of ewes and if decreased from late winter up to summer. The rams also showed a decrease in vitamin E concentration from late winter to summer. The variation was higher in rams than in ewes (Table 4). Higher values were found in rams from September up to late winter. The observed increase from early autumn to late winter coincided with changes in the sexual activity of ewes and rams, while the requirement due to the cold season was also important. The observed pattern is different from that reported for cattle (Hidiroglou and Williams, 1986), and the mechanisms for α-tocopherol absorption or excretion differ between sheep and cattle (Caravaggi, 1969; Hidiroglou and Williams, 1986).

Hidiroglou (1983) reported that the date was a significant ($P < 0.01$) source of variation for vitamin E concentration in sheep blood plasma.

Table 4

Vitamin A and E concentration of the blood plasma of sheep during the year (n = 6)

Month	Ewes				Rams			
	Vitamin A μg/L		Vitamin E μg/L		Vitamin A μg/L		Vitamin E mg/L	
	mean	SEM	mean	SEM	mean	SEM	mean	SEM
April	455	14	1.30	0.49	442	13	1.30	0.49
May	409	19	1.19	0.79	420	11	1.13	0.49
June	462	5	1.13	0.14	446	9	1.28	0.46
July	456	18	0.87	0.35	464	9	1.15	0.64
August	448	10	1.07	0.37	478	8	1.23	0.53
September	451	11	1.17	0.53	471	10	0.94	0.34
October	471	10	1.33	0.48	465	7	1.04	0.21
November	479	5	1.32	0.52	478	8	1.24	0.33
December	450	8	1.09	0.52	463	4	0.99	0.28
January	485	12	1.18	0.46	474	10	1.33	0.50
February	446	9	1.14	0.59	476	10	1.59	0.80
March	444	11	1.41	0.24	433	9	1.22	0.34

SEM: standard error of mean

In general, vitamin E concentration of the blood plasma showed higher variations than vitamin A. The concentration of vitamin E, as compared to that of vitamin A, seems to be markedly influenced by feed intake, feed content and/or temperature variations. The vitamin E reservoirs in the body are limited as compared to vitamin A and are more susceptible to environmental factors such as season and feed intake. Judson et al. (1991) also reported that a vitamin E concentration of 120 mg/kg diet was required for significantly increasing and maintaining liver reservoirs for up to 2 months, using a ration containing a low level of vitamin E. In the present study, a stardardized vitamin E supply was used; for that reason, temperature (season) and sexual activity had the most expressed effect on the changes as were found during the period of investigation. The results also indicate that the traditional Iranian Shal breed of sheep reacts to environmental and other factors in more or less the same way as was described for sheep of the Merino breed.

Table 5

Daily vitamin A and E intake of sheep during the year

Month	Ewes		Rams	
	β-carotene mg/animal	Vitamin E mg/animal	β-carotene mg/animal	Vitamin E mg/animal
April	17.69	28.85	20.70	29.55
May	17.16	29.52	18.40	30.16
June	16.20	26.16	20.36	26.77
July	16.45	28.41	19.25	29.03
August	17.66	25.75	20.98	26.25
September	18.70	29.97	21.85	30.51
October	17.08	28.08	19.90	28.64
November	15.08	26.53	17.70	26.99
December	16.50	24.97	18.70	25.51
January	17.37	28.64	21.33	29.12
February	20.85	30.19	19.81	30.77
March	17.86	29.30	23.90	29.90

Acknowledgements

This work was supported by the Ministry of Construction Jihad of I. R. Iran, Deputy of Education and Research. The support, technical guidance and assistance provided by M. Khorvash, G. Nagy and A. Abbasi are gratefully acknowledged.

References

A.O.A.C. (1990): Official Methods of Analysis, 15th edition., AOAC, Washington, D. C.

Bedő, S., Keszthelyi, T., Mézes, M. and Jammoul, A. (1992): Seasonal variation in vitamin A and E content of blood plasma of ewes of differing genotypes (in Hungarian, with English abstract). Állattenyésztés és Takarmányozás **41,** 153–163.

Biesalski, H., Greiff, H., Brodda, K., Hafner, G. and Bässler, H. (1986): Rapid determination of vitamin A (retinol) and vitamin E (α-tocopherol) in human serum by isocratic adsorption HPLC. Int. J. Vit. Nutr. Res. **56,** 319–327.

Caravaggi, C. (1969): Vitamin E concentration in the serum of various experimental animals. Comp. Biochem. Physiol. A **30,** 585–588.

Chow, F. I. and Omaye, S. T. (1983): Use of antioxidants in the analysis of vitamins A and E in mammalian plasma by high performance liquid chromatography. Lipids **18,** 837–841.

Glover, J. C. J., Kershaw, R. C. and Reilly, P. E. B. (1976): Seasonal changes in the plasma retinol-binding holoprotein concentration in sheep. Br. J. Nutr. **36,** 137–141.

Hidiroglou, M. (1983): Blood plasma minerals and vitamins and acid-base status of sheep raised under fluctuating or constant environment. J. Dairy Sci. **66,** 67–72.

Hidiroglou, M. and Williams, C. J. (1986): Interrelationships among liposoluble vitamins in ruminants. Am. J. Vet. Res. **8,** 1767–1771.

Judson, G. J., Babidge, P. J. and Babidge, W. J. (1991): Plasma, liver and fat α-tocopherol concentrations in sheep given various oral and subcutaneous doses of vitamin E. Australian J. Exp. Agric. **31,** 45–50.

McMurray, C. H. and Blanchflower, W. J. (1979): Determination of α-tocopherol in animal feedstuffs using high performance liquid chromatography with spectrofluorescence detection. J. Chromatogr. **176,** 488–492.

Molokwu, E. C. I. (1978): Seasonal changes in bovine and caprine blood chemistry and hepatic vitamin A in savanna zone of Nigeria. Br. Vet. J. **134,** 493–500.

Thompson, J. N. (1969): The role of vitamin A in reproduction. In: DeLuca, H. F. and Suttie, J. W. (eds) The Fat Soluble Vitamins. Univ. Wisconsin Press, Madison, pp. 267–281.

Wooton, I. D. P. (1962): Normal variation in blood constituents. Proc. Nutr. Soc. **21,** 129.

Acta Veterinaria Hungarica 43 (4), pp. 463–473 (1995)

VARIATIONS IN THE YIELD, COMPOSITION AND SOMATIC CELL COUNT OF EWE'S MILK DURING LACTATION

S. BEDŐ[1], Etelka NIKODÉMUSZ[1] and Katalin GUNDEL[2]

[1]Institute of Animal Husbandry, Gödöllő University of Agricultural Sciences, H–2103 Gödöllő, Páter K. u. 1, Hungary; [2]National Institute for Breeding Performance Testing, H–2100 Gödöllő–Nagyremete, Hungary

(Received February 12, 1995)

Variations in the yield, composition and somatic cell count of milk were studied in 30 grazing ewes, representing the Pleven F_1 × East-Friesian Blackface, Pleven F_1 and Merino genotypes, during five months of lactation. The somatic cell count (SCC) correlated negatively with milk yield and lactose whilst positively with milk protein and milk fat, except for Pleven F_1 ewes which showed these correlations with reversed values. The highest milk yield with the lowest SCC was shown by the Pleven F_1 × East-Friesian Blackface genotype. Lactose concentration varied in a significant negative correlation with the neutral detergent fibre content of the grass.

Key words: Ewes, genotype, grass, lactation, lactose, milk fat, milk protein, milk yield, somatic cell count

The somatic cell count (SCC) in ewe's milk may vary greatly and an accepted normal value has not been established (Ranucci and Morgante, 1994). On the present hygienic level of ewe's milk production a value of 1.0×10^6 cells/ml can be suggested as the upper limit for normal ewe's milk (Green, 1984; Fthenakis et al., 1991).

Numerous studies, mostly on cows' milk, show that an increase in SCC may interfere with the chemical composition and properties of milk.

The increased attention attracted recently by ovine milk has prompted studies on the hygienic and compositional quality of ewe's milk during lactation (Anifantakis et al., 1994; Bufano et al., 1994; Kukovics et al., 1994; Margetin et al., 1994; Ubertalle et al., 1994).

Variations in the yield, composition and somatic cell count of the milk of 30 ewes representing three genotypes during five months of lactation are presented in this paper.

0236–6290/95/$ 5.00 © 1995 *Akadémiai Kiadó, Budapest*

Materials and method

Animals. Ten ewes of the Pleven F_1 × East-Friesian Blackface, Pleven F_1 (Merino × Pleven Blackface) and the Merino genotypes each were examined in the Délborsod Fishing and Sheep-Farming Cooperative at Gelej during 1994. Animals were randomly selected and identified by ear-tags. Ewes lambed in late February and were lactating till mid-August.

Table 1

Variations in the dry matter (DM) base composition of feeds provided to ewes during lactation

Month	Feed	(n)	DM	CP	CF	CFib
			g/kg	g/kg DM		
1 (April)	Grass	(3)	176.0	143.6	39.1	183.0
			±46.7	±25.9	±8.7	±31.8
	Corn	(1)	907.8	96.5	38.7	21.5
2 (May)	Grass	(3)	255.0	148.2	26.2	224.6
			±51.3	±20.5	±5.7	±28.1
	Corn	(1)	832.5	85.3	38.9	20.9
3 (June)	Grass	(3)	439.0	115.7	24.7	279.9
			±48.0	±0.7	±1.6	±13.7
	Corn	(1)	900.1	91.0	38.9	18.2
	Concentrate*	(1)	932.7	388.5	10.4	67.0
4 (July)	Grass	(3)	756.0	78.5	23.7	320.9
			±117.5	±20.9	±3.1	±13.8
	Corn	(1)	915.6	93.2	35.7	19.8
	Peas**	(1)	916.9	248.9	13.0	59.9
	Concentrate*	(1)	948.7	386.7	10.5	73.9
5 (August)	Grass	(3)	933.0	58.3	17.9	348.0
			±0.2	±4.8	±2.2	±6.8
	Corn	(1)	914.8	67.7	35.5	22.4

The nutrient composition of feeds was determined according to Hungarian Standard no. MSZ 6830/14-78

DM = dry matter, CP = crude protein, CF = crude fat, CFib = crude fibre; * Pleven F_1 × East-Friesian Blackface; ** Merino, Pleven F_1

Feeds. The staple diet was grass grazed by ewes. Based on samplings, the estimated grass yield of the pasture was 3480 kg/ha in May, 2920 kg/ha in June and 740 kg/ha in August. Grass was supplemented with corn in a daily portion of 0.6 kg/ewe. Besides, Pleven F_1 × East-Friesian Blackface ewes were provided

with a concentrate (0.6 kg/ewe) in months 3–4 of lactation and the two other genotypes with peas (0.3 kg/ewe) in month 4. Variations in the dry matter (DM) base composition of feeds are given in Table 1.

Milk analysis. After the suckling period ewes were milked by machine. Evening and morning milk samples were individually taken and milk yields (MY) recorded at 4-week intervals. (Except for the replacement of 1–2 ewes in months 2 and 3 due to intercurrent injuries or death, resp., the same individuals were sampled.) Preserved (Bromopole) milk samples were analyzed for milk fat (MF), milk protein (MP), lactose (LC) and SCC using a Combi-Foss device (Foss Electric, Denmark).

Data analysis. Means ± S.D. values were compared by Student's t-test. The correlations between SCC and MY, MP, LC were determined by simple regression as outlined in STATGRAPHICS (STSC Inc. and Statistical Graphics Corp., 1985).

Results

With the exception of MF that was significantly higher ($P < 0.01$ and $P < 0.001$) in the evening samples, no significant diurnal variations were noted in the variables. Thus, the mean values for the individual morning and evening milk samples were taken.

The lactation curves of ewes showed a characteristic decreasing pattern (Fig. 1).

The variations in MF, MP and LC during lactation are illustrated in Figs 2–4. All three genotypes showed significant increases in MF ($P < 0.001$ and $P < 0.05$) and MP ($P < 0.05$ and $P < 0.1$) whilst significant decreases in LC ($P < 0.01$ and $P < 0.001$) through months 3–5.

The SCC curve was U-shaped in Pleven F_1 × East-Friesian Blackface ewes, it took an increasing course in Merinos and a definitely decreasing one in Pleven F_1 ewes (Fig. 5). The between-month differences in SCCs were non-significant.

Based on a regression analysis of data, the SCC correlated negatively with MY and LC whilst positively with MP and MF, except in Pleven F_1 ewes which showed these correlations with reversed values (Table 2).

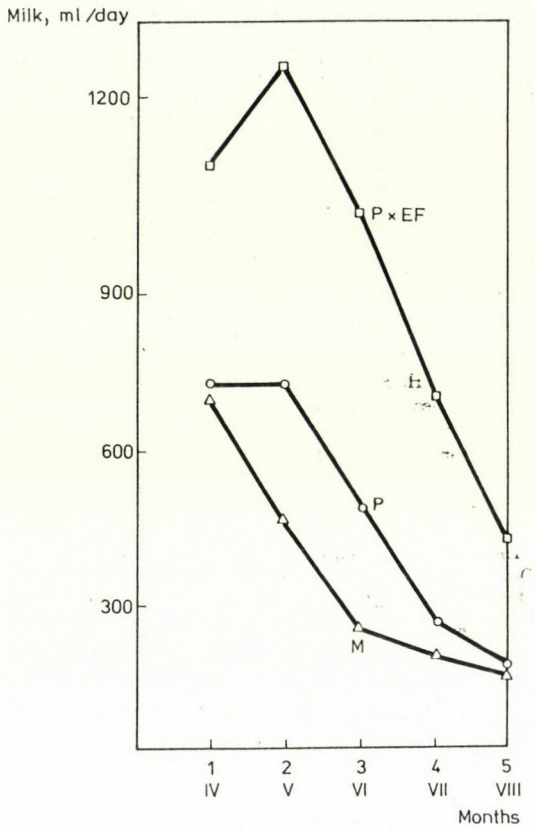


Fig. 1. Lactation curves of ewes (P × EF=Pleven F_1 × East-Friesian Blackface, overall S.D.=442; P=Pleven F_1, overall S.D.=326; M=Merino, overall S.D.=279). Each point represents 10 samples, except for Pleven F_1 in month V (n=8) and for Merino in months IV–V (n=6 and 5, respectively)

Discussion

Our sample revealed considerable differences in the variables according to genotype.

Pleven F_1 × East-Friesian Blackface ewes showed the highest MY and LC whilst Merinos produced the highest MF and MP. Pleven F_1 ewes represented intermediate values. The differences are partly due to the surplus dietary protein given to Pleven F_1 × East-Friesian Blackface ewes in months 3–4 and partly to genetic differences (the cross-bred genotypes are of milking-type). According to reported data, ewes of the Pleven Blackface and Hungarian Merino × East-

Friesian genotypes produced milk in significantly ($P < 0.01$) higher quantities and also utilized dietary energy and crude protein more efficiently than the Merino breed (Bedő et al., 1989).

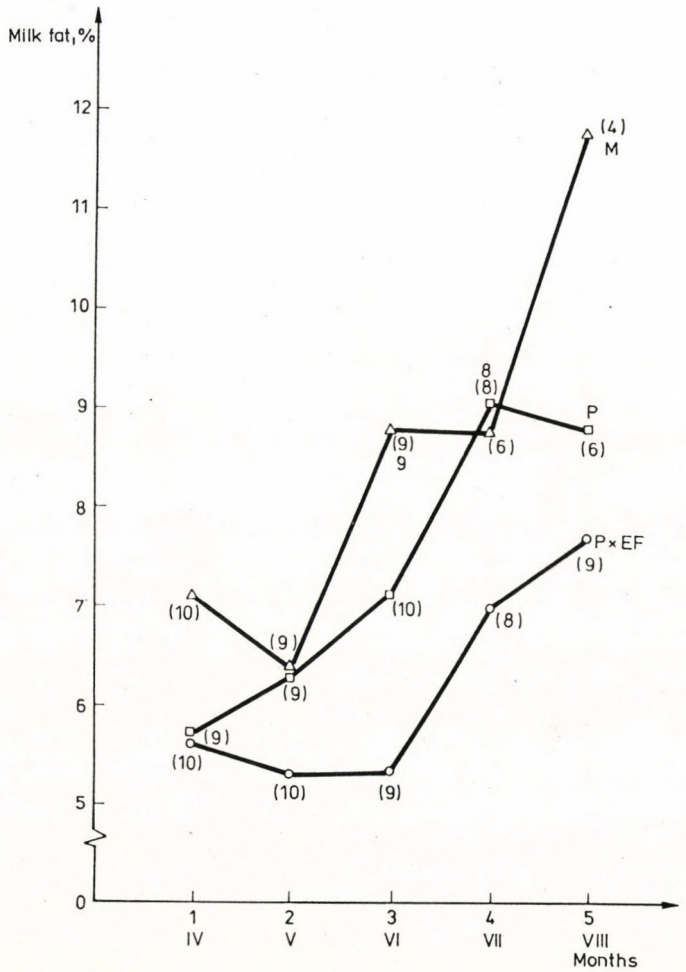


Fig. 2. Variations in milk fat values of ewes during lactation. P × EF=Pleven F_1 × East-Friesian Blackface, overall S.D.=1.43; P=Pleven F_1, overall S.D.=1.55; M=Merino, overall S.D.=2.01. Figures in parentheses indicate the number of samples

For the whole lactation period, the SCCs were below the suggested threshold value of 1.0×10^6 cells/ml in Pleven F_1 × East-Friesian Blackface ewes and Merinos and were only surpassed by those of Plevens during months 1–2. However, the individual variations were high due to the small sample size. The

SCC curve of Pleven F_1 × East-Friesian Blackface ewes resembled to that of cows (Nikodémusz et al., 1994*a*), while the SCC curve of Merinos was similar to those reported for the Greek ewe breeds (Anifantakis et al., 1994).

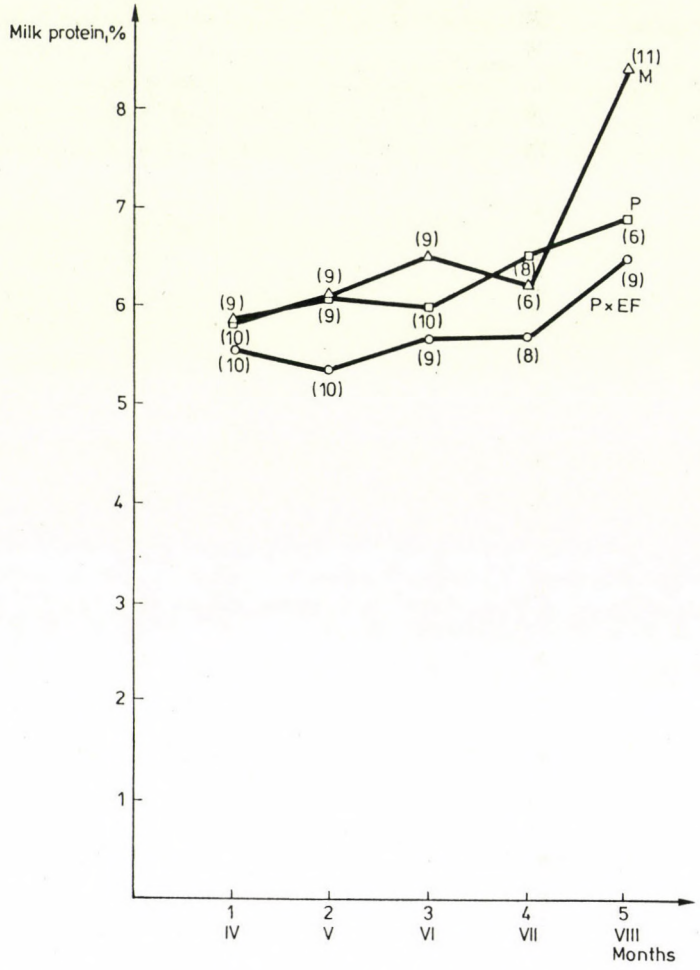


Fig. 3. Variations in milk protein values of ewes during lactation. P × EF=Pleven F_1 × East-Friesian Blackface, overall S.D.=0.68; P=Pleven, overall S.D.=0.88; M=Merino, overall S.D.=1.05. Figures in parentheses indicate the number of samples

In good agreement with data reported for other ewe breeds (Kukovics et al., 1994; Margetin et al., 1994; Ubertalle, 1994) the SCC correlated negatively with MY and LC, and it correlated positively with MP and MF during lactation in all genotypes except the Pleven.

Lactose content was less stable than it was normally expected to be: it decreased significantly in all three genotypes through months 4–5 (Fig. 4).

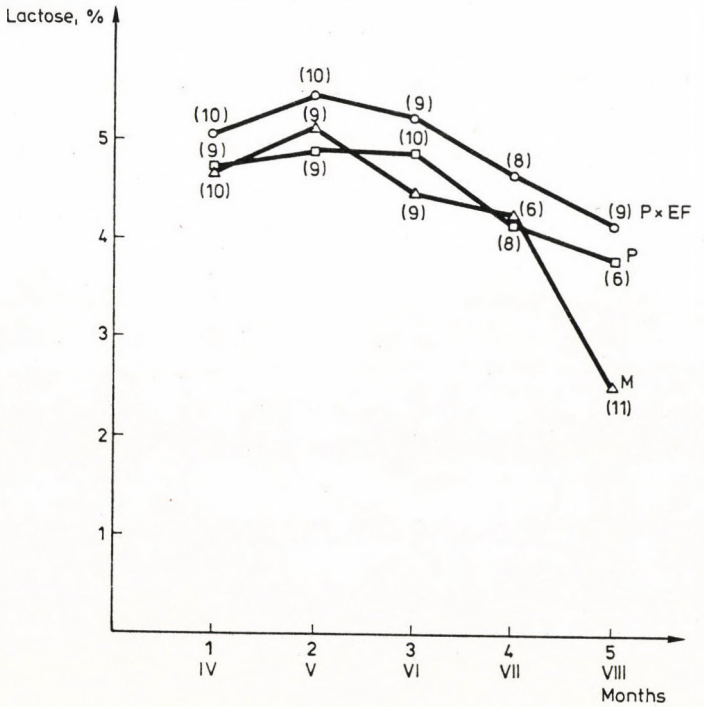


Fig. 4. Variations in the lactose values of ewes during lactation. P × EF=Pleven F_1 × East-Friesian Blackface, overall S.D.=0.54; P=Pleven F_1, overall S.D.=0.61; M=Merino, overall S.D.=0.91. Figures in parentheses indicate the number of samples

This period coincided with the hot season, characterized by an average daily air temperature of 23–24 °C with maximums of 35–36 °C, prevailing here from mid-June to mid-August.

The droughty weather led to early aging of the grass: crude protein (CP) decreased by 50 %, while crude fibre (CFib) and dry matter (DM) increased by 20 % and 53 %, respectively between months 3 and 5 of lactation (Table 1). The ratio of neutral detergent fibre (cell walls) to neutral detergent solubles (cell contents) increased 5.5-fold (Fig. 6).

Crude protein deficiency was compensated for by providing a concentrate or peas containing 390 g and 250 g of CP/kg DM, respectively. However, the deficiency in the soluble carbohydrates (sugars, starch) of the grass could lead to a drop in the ewes' blood glucose concentration. Consequently, the secretion of lac-

tose and water (milk production) also decreased as a result of a reduced energy production from glucose by the tricarboxylic acid (TCA) cycle, so as to buffer the osmotic pressure of milk to that of blood (Kállai and Kralovánszky, 1975; Giesecke et al., 1990).

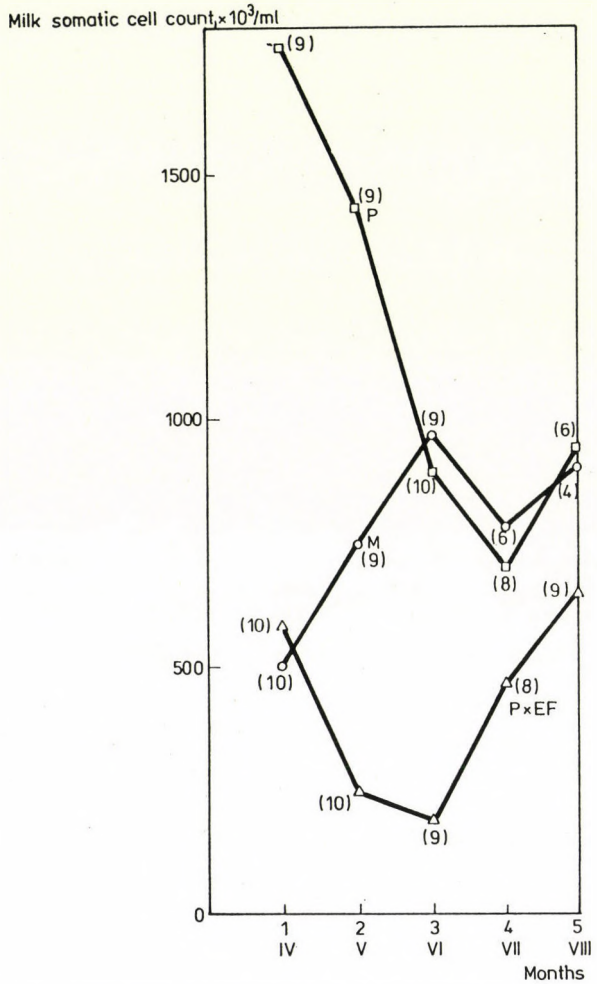


Fig. 5. Variations in milk somatic cell count of ewes during lactation. P × EF=Pleven F_1 × East-Friesian Blackface, overall S.D.=542; P=Pleven F_1, overall S.D.=1740; M=Merino, overall S.D.=779. Figures in parentheses indicate the number of samples

The significant negative correlation between NDF and LC in each genotype ($r = -0.90$, $P < 0.05$) confirms this hypothesis. It also gains support from the significant decreases found in the lactose concentrations of ewes' and goats' milk

in Greece during the last two months of lactation, coinciding with the summer heat (Anifantakis et al., 1994).

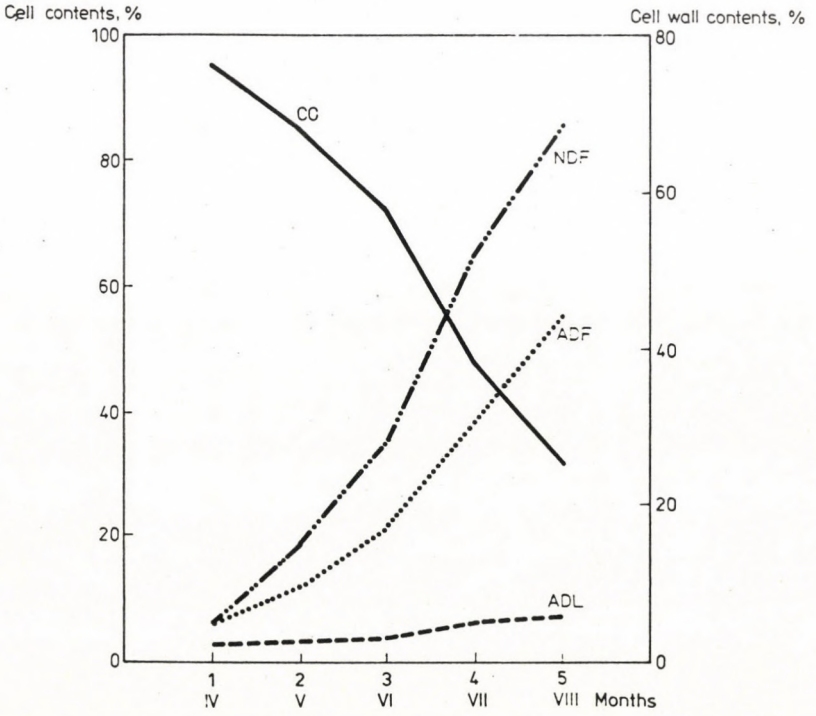


Fig. 6. Variations in the cell contents and cell wall contents of the grass grazed by ewes during lactation. C=cell contents, cell wall contents; NDF=neutral detergent fibre, ADF=acid detergent fibre, ADL=acid detergent lignin. The fibre fractions were determined according to Goering and Van Soest (1970)

In this study, the summer heat also produced significant depressions in the number of circulating leucocytes with a right shift in the ratio of lymphocytes to neutrophils (from 3.2:1 to 4.0:1) between months 3 and 5 (Nikodémusz et al., 1994*b*). This might have interfered with, or lowered, the late-lactation SCC scores in ewes, especially in Plevens that also showed a drop in red blood cell count and packed cell volume during the last month.

In grazing goats, the SCC of milk tended to decrease as the fibre intake increased and, on the contrary, it showed an increase as the nitrogen balance became more and more positive (Fedele et al., 1994).

Table 2

Correlations observed between SCC and the test variables according to genotype

Variable	Total sample			Monthly means		
	Pleven F_1 × East-Friesian (n=46) r	Pleven F_1 (n=42) r	Merino (n=38) r	Pleven F_1 × East-Friesian (n=5) r	Pleven F_1 (n=5) r	Merino (n=5) r
MY	–0.21	0.19	–0.15	–0.64	0.88**	–0.85**
LC	–0.44***	–0.01	–0.06	–0.79*	0.54	–0.48
MP	0.57****	–0.04	0.22	0.63	–0.66	0.59
MF	0.27*	–0.21	0.10	0.72	–0.87**	0.60

Monthly means = means of samples evaluated per genotype during months 1–5 of lactation. The corresponding n values were: 10, 10, 9, 8, 9, for Pleven F_1 × East-Friesian Blackface ewes, 10, 9, 9, 8, 6 for Plevens and 9, 9, 10, 6, 4 for Merinos. Asterisks indicate the significance levels: $P < 0.1$; $P < 0.05$; $P < 0.01$; $P < 0.001$

References

Anifantakis, E. M., Vastardis, J., Gardika, A., Giannakopoulou, K., Fiskatoris, M. and Karavassilis, C. (1994): A survey of raw ewe's and goat's milk quality in Greece. Int. Symp. "Somatic Cells and Milk of Small Ruminants", Bella, Italy. 25–27 September 1994. Ses. 4, pp. 1–20.

Bedő, S., Barcsák Tóth G., Ferenczy Lévay, M., Póti, P. and Bihari, Gy. (1989): Utilization of nutrients for milk production by ewes of different genotypes (in Hungarian). Állattenyésztés és Takarmányozás **38**, 425–438.

Bufano, D., Dario, C. and Laudadio, V. (1994): The characterizing of Leccese sheep: Variations of chemical composition and lactodynamographic parameters in milk as related to somatic cell count. Int. Symp. "Somatic Cells and Milk of Small Ruminants", Bella, Italy. 25–27 September 1994. Ses. 3, pp. 39–42.

Fedele, V., Claps, S. and Rubino, R. (1994): Effect of feeding systems on somatic cell counts in goats. Int. Symp. "Somatic Cells and Milk of Small Ruminants", Bella, Italy. 25–27 September 1994. Ses. 2, pp. 25–29.

Fthenakis, G. C., El-Massanat, E. T. S., Both, J. M. and Jones, J. E. T. (1991): Somatic cell counts of ewes' milk. Br. Vet. J. **147**, 575–581.

Giesecke, W. H., Korybut-Woroniecki, P. A. and Kowalski, Z. E. (1990): Udder health implications of premature bovine mammary regression. I. Clinical, subclinical and reducing-sugar changes in milk during 168 hours of suspended milking in mid-lactation. Onderstepoort J. Vet. Res. **57**, 25–35.

Goering, H. K. and Van Soest, P. J. (1970): Forage fiber analyses (apparatus, reagents, procedures and some applications). U.S. Dept. Agric. Handb. 379. 20 pp.

Green, T. J. (1984): Use of somatic cell counts for detection of subclinical mastitis in ewes. Br. Vet. J. **143**, 402–409.

Kállai, L. and Kralovánszky, U. P. (1975): Biology of Meat and Milk production (in Hungarian). Mezőgazdasági Kiadó, Budapest, pp. 201–207.

Kukovics, S., Molnár, A. and Ábrahám, M. (1994): The somatic cells situation of small ruminants in Hungary. Int. Symp. "Somatic Cells and Milk of Small Ruminants", Bella, Italy. 25–27 September 1994. Ses. 4, pp. 41–45.

Margetin, M., Capistrak, A., Kica, J., Valkovsky, P. and Foltys, V. (1994): Somatic cell count, production and milk composition in sheep up to weaning and after it. Int. Symp. "Somatic Cells and Milk of Small Ruminants", Bella, Italy. 25–27 September 1994. Ses. 2, pp. 27–32.

Nikodémusz, E., Bedő, S., Pichler, A. and Szép, P. (1994*a*): Variations in milk somatic cell count and haematological values of dairy cows during lactation. Acta Vet. Hung. **42,** 131–139.

Nikodémusz, E., Bedő, S. and Gundel, K. (1994*b*): Variations in milk somatic cell count and haematological values of ewes during lactation. Int. Symp. "Somatic Cells and Milk of Small Ruminants", Bella, Italy. 25–27 September 1994. Ses. 2, pp. 46–50.

Ranucci, S. and Morgante, M. (1994): Sanitary control of the sheep udder: total and differential cell counts in milk. Int. Symp. "Somatic Cells and Milk of Small Ruminants", Bella, Italy. 25–27 September 1994. Ses. 1, pp. 1–8.

STSC Inc. and Statistical Graphics Corp. (1985): STATGRAPHICS Statistical Graphics System. Version 4.0. Licensed Software, USA.

Ubertalle, A., Battaglini, L. M., Fortina, R. and Bianchi, M. (1994): Effect of some variation factors on somatic cell count in Delle Langhe sheep milk. Int. Symp. "Somatic Cells and Milk of Small Ruminants", Bella, Italy. 25–27 September 1994. Ses. 2, pp.17–22.

MAGYAR
TUDOMÁNYOS AKADÉMIA
KÖNYVTÁRA

COMING EVENT

FOOD MICRO '96

TECHNOLOGY, SAFETY, STABILITY

16th International Symposium of the International Committee
on Food Microbiology and Hygiene
(IUMS–ICFMH)

First Announcement

27–30 August 1996
University of Horticulture and Food

Budapest
HUNGARY

Invitation

The Organizing Committee cordially invites you to participate in the 16th International Symposium "Food Micro '96", held at the University of Horticulture and Food, Budapest, Hungary, 27–30 August, 1996. The symposium will be organized under the auspices of the International Committee on Food Microbiology and Hygiene, the Hungarian Scientific Society for Food Industry and the University of Horticulture and Food.

Scientific Committee

Deák, T. (Chairman), Budapest; Corry, J. E. L., Langford; Curtis, G. W. D., Oxford; Farkas, J., Budapest; Holzapfel, W., Karlsruhe; Incze, K., Budapest; Jakobsen, M., Copenhagen; Kiss, I., Budapest; Mossel, D. A. A., Utrecht; Ralovich, B., Budapest; Rombouts, F. M., Wageningen; Sas, B., Budapest; Skovgaard, N., Copenhagen

Local Organizing Committee

Kiss, I. (Chairman); Bánáti, D.; Fábri, I.; Hernádi, Z.; Hoschke, Á.; Kovács, S.; Krász, Á.; Maráz, A.; Mohácsi-Farkas, Cs.; Rodler, M.; Steiner, A.; Szentpéteri, K.; Szita, G.; Tabajdi-Pintér, V.

Scientific programme

Objectives of the Symposium are to present and discuss effects of microbiological quality of raw materials, handling and processing technologies on safety and microbiological stability of foods, evaluation of microbiological methods, prediction of safety and shelf life, and new informations on foodborne pathogens as well.

Topics

1. Microbiological quality of raw materials
2. Hygiene, stability (shelf-life), technology
3. Microbiological safety of new food processing methods
4. Food biotechnology. Biopreservation
5. Combined treatments. Interactions of hurdles
6. Rapid methods (monitoring, automatization)
7. Predictive microbiology
8. Culture media and their quality control

The program will consist of invited lectures, contributed papers and poster presentations.
Those intending to deliver a paper or present a poster are kindly requested to send a draft abstract (not more than 100 words) to the Congress Office, indicated below.
The Congress Office will confirm the acceptance of oral and poster presentations.

Language

English will be the language of the Symposium.

Site of Symposium

University of Horticulture and Food
Villányi út 35–43, Budapest
H–1118 HUNGARY

Congress Office

Meeting Budapest
Dutch-Hungarian Organizer and Consulting Ltd.
H–1027 Budapest, Csalogány u. 23–25.

"FOOD MICRO '96" SECRETARIAT
Phone: (36–1) 212–1667
(36–1) 201–2577
Fax: (36–1) 212–2623

ANNOUNCEMENT

IAMS Company Award 1995

Veterinary science is a rapidly developing field of knowledge. Veterinary practitioners have to rely on high standard publications in Veterinary Journals to keep up with the new developments in their profession. The IAMS Company is at the forefront of the new science in Companion Animal Science and considers it her obligation to make this science available to the Veterinary Community. The Company wants to express its support for outstanding veterinary publications by awarding the best article that was published between 1 January 1994 and 1 July 1995.

Over 40 outstanding articles were submitted by authors from all over Europe to compete for the 1995 IAMS Company Award. The independent committee of three well known clinicians working at European Veterinary Faculties, decided unanimously that the 1995 IAMS Company Award should be given to Dr. Peter Srenk from Brno, Czech Republic, for his publication "Genetical aspects of idiopathic epilepsy in the Golden Retriever" which was published in the Tierärztliche Praxis 22: 574–578 (1994). The study was carried out at the University of Bern, Switzerland. The Award, ECU 2,500.00 cash, plus travel and congress arrangements, was presented at the second FECAVA Congress which was held in Brussels 27 through 29 October 1995.

In the winning publication Dr. Srenk and his co-authors from the Veterinary Faculty in Bern describe a study in 336 pedigrees of normal and epileptic Golden Retrievers. A significant predisposition for males was found. The results support the hypothesis of an autosomal multifactorial recessive mode of inheritance. The article will be reprinted in the 1996 spring issue "The European Journal of Companion Animal Practice", the official journal of the FECAVA.

The participation in the competition for the IAMS Company Award 1996 is open for junior veterinarians (35 years and under) who have published important clinical work in a European Veterinary Journal between July 1st 1995 and July 1st 1996. For information please contact IAMS Company Award Committee secretary Mrs. M. v.d. Kraan, IAMS Pet Food International Inc., Luchthavenweg 67, NL–5657 EA Eindhoven, The Netherlands.

PRINTED IN HUNGARY

Akadémiai Kiadó és Nyomda, Budapest

Acta Veterinaria
Hungarica

EDITOR-IN-CHIEF

J. MÉSZÁROS

ASSOCIATE EDITOR

A. SZÉKELY

EDITORIAL BOARD

**A. BARTHA, J. BOKORI, T. KASSAI, F. KUTAS,
G. PETHES, P. RUDAS, L. SOLTI, S. TUBOLY,
J. VARGA, F. VETÉSI, K. VÖRÖS**

VOLUME 43

AKADÉMIAI KIADÓ, BUDAPEST
1995

Acta Veterinaria Hungarica

VOLUME 43

CONTENTS

Animal nutrition

Complex study of the physiological role of cadmium I. Cadmium and its physiological role. *Bokori, J.* and *Fekete, S.* 3

Complex study of the physiological role of cadmium II. Effect of cadmium load on the cadmium content of eggs. *Bokori, J., Fekete, S., Kádár, I.* and *Albert, M.* 45

Complex study of the physiological role of cadmium III. Cadmium loading trials on broiler chickens. *Bokori, J., Fekete, S., Kádár, I., Koncz, J., Vetési, F.* and *Albert, M.* 195

Effect of avoparcin on rumen fermentation and duodenal nutrient flow in sheep. *Fébel, Hedvig, Romváry, A., Zsolnai-Harczi, Ildikó* and *Huszár, Szilvia* 229

Animal reproduction

In vitro and *in vivo* motility studies of ^{99m}Tc HM-PAO labelled sperm cells. *Balogh, L., Szász, F., Zöldág, L., Huszenicza, Gy., Tóth, L., Dabasi, Gabriella* and *Jánoki, Gy.* 63

Effects of sample handling temperatures on bovine skim milk progesterone concentrations. *Eissa, H. M., Nachreiner, R. F.* and *Refsal, K. R.* 79

Effect of luteinizing hormone and estradiol on *in vitro* maturation of follicular oocytes in goat (*Capra hircus*). *Sidhu, K. S.* and *Cheema, S.* 89

Seasonal changes in sperm parameters of British milk rams. *Sarlós, P.* and *Molnár, A.* 247

Successful embryo recovery from swine by a minimal invasive technique. *Rátky, J.* and *Brüssow, K.-P.* 371

Bacteriology

The production of K88 antigen by *Escherichia coli* and *Salmonella typhimurium* strains with recombinant DNA. *Holoda, E.* and *Mikula, I.* 95

Epizootiology

Achievements and difficulties in maintaining the tuberculosis-free status of Hungarian cattle herds. *Körmendy, B.* 377

The value of immunodiagnostic tests in detecting tuberculosis in an infected red deer herd and in eradication of the disease by selection. *Zomborszky, Z., Körmendy, B., Tuboly, S., Tilly, P.* and *Horn, P.* 385

Immunology

Intestinal absorption of colostral lymphocytes in newborn lambs and their role in the development of immune status. *Tuboly, S., Bernáth, S., Glávits, R., Kovács, Andrea* and *Megyeri, Z.* 105

Mycotoxin research

Contamination of broiler chicken's mash and litter with moulds, aflatoxins, ochratoxin A and zearalenone. *Škrinjar, M., Ristić, M.* and *Grbić, Z.* 117
Occurrence of *Fusarium* species and zearalenone in dairy cattle feeds in Vojvodina. *Škrinjar, M., Stubblefield, R. D., Stojanović, E.* and *Dimić, G.* 259
Detection of ochratoxin A in human blood and colostrum. *Kovács, F., Sándor, Gabriella, Ványi, A., Domány, S.* and *Zomborszky-Kovács, Melinda* 393

Parasitology

Histological changes in the swimbladder wall of eels due to abnormal location of adults and second stage larvae of *Anguillicola crassus. Molnár, K., Szakolczai, J.* and *Vetési, F.* 125
Ultrastructural observations on the third-generation merozoites of *Eimeria tenella* in chicks. *Ball, S. J., Daszak, P., Pittilo, R. M.* and *Norton, C. C.* 139
Redescription of *Goussia neglecta* n. comb. (Nöller, 1920) (Apicomplexa; Coccidia) and notes on its occurrence in the gut of tadpoles. *Molnár, K.* 269
Effect of exposure to malachite green solution on common carp fry with *Dactylogyrus vastator* (Monogenea) infection. *Molnár, K.* 277
Prevalence of botfly larvae and lice in studs of North Caucasus (Stawropol County, Russia). *Egri, B., Sárközy, P.* and *Bánhidy, Gy.* 287
Dynamics of *Anguillicola crassus* (Nematoda: Dracunculoidea) larval infection in paratenic host fishes of Lake Balaton, Hungary. *Székely, Cs.* 401

Pathology

Eosinophils in lymph nodes of cows infected by bovine leukaemia virus. *Levkut, M., Levkutová, Maria, Konrád, V.* and *Poláček, M.* 145
Effect of mercury on the seminiferous epithelium of the fowl testis. *Maretta, M., Marettová, E., Škrobánek, P.* and *Ledeč, M.* 153

Pathophysiology

Betamethasone and adult rat lung surfactant lipids. *Stettner, S.* and *Ledwożyw, A.* 291
The effect of betamethasone on phosphatidylcholine species composition in fetal rat lungs. *Stettner, S.* and *Ledwożyw, A.* 297
Prenatal starvation, betamethasone and lung development in newborn rats. *Stettner, S.* and *Ledwożyw, A.* 303
Protective effect of Verapamil on regional myocardial ischaemic injury in dog. *Kobusiewicz, W., Ogonowska-Kobusiewicz, Maria, Michalak, J., Zywicki, W.* and *Ledwożyw, A.* 311

MTA Könyvtára
Példányszám 12. 96/971

Evaluation of blood lipid peroxidation parameters in carbon tetrachloride (CCl_4) toxicity in sheep. *Vajdovich, P., Szilágyi, A.* and *Gaál, T.* 423
Effects of histamine on gizzard erosions and on the activity of selected enzymes in chickens. *Džaja, P., Grabarević, Ž., Perić, J., Artuković, Branka, Tišljar, Marina, Mrljak, V.* and *Šoštarić, Edita* 431

Physiology

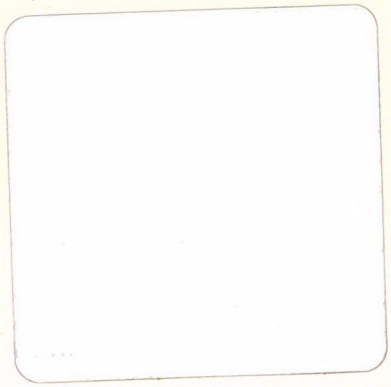
Variations in the milk yield and milk composition of dairy cows during lactation. *Bedő, S., Nikodémusz, Etelka, Percsich, K.* and *Bárdos, L.* 163
Effect of severe energy restriction and refeeding on thyroid hormones in bulls. *Janan, J., Rudas, P., Bartha, T., Bozó, S.* and *Gábor, Gy.* 173
Effect of hydrothermal treatment of rice straw on its composition, *in sacco* digestibility and *in vitro* fermentation by rumen microorganisms. *Adya, M., Sareen, V. K.* and *Singh, Sudarshan* 179
Differences in the thryroxine, triiodothyronine and reverse triiodothyronine contents of fetal pig tissues relative to gestational age. *Krysin, Ewa* 443
Seasonal variation in the concentration of vitamins A and E in the blood plasma of fat-tailed sheep. *Asadian, S., Mirhadi, S. A.* and *Mézes, M.* 453
Variations in the yield, composition and somatic cell count of ewe's milk during lactation. *Bedő, S., Nikodémusz, Etelka* and *Gundel, Katalin* 463

Toxicology

Toxicological studies on potentiated ionophores in chickens I. Tolerance study. *Lehel, J., Laczay, P., Móra, Zsuzsa* and *Semjén, G.* 321
Toxicological studies on potentiated ionophores in chickens II. Compatibility study. *Lehel, J., Laczay, P., Móra, Zsuzsa* and *Semjén, G.* 335
Toxicological studies on potentiated ionophores in chickens III. Electrotoxicological investigations. *Lehel, J.* and *Laczay, P.* 347
Effects of repeated oral doses of Dikamin D (2,4-D-amine Na) on rats. *Várnagy, L., Somlyay, I., Budai, P.* and *Varga, T.* 355
Experimental furazolidone toxicosis in broiler chicks: Effect of dosage, duration and age upon clinical signs and some blood parameters. *Zaman, Q., Khan, M. Z., Islam, N.* and *Muhammad, G.* 359

Coming Event 191
Coming Event 475

Announcement 477



INSTRUCTIONS TO AUTHORS

Manuscripts are accepted on the basis of scientific significance and suitability for publication on the understanding that they have not been published, submitted or accepted for publication elsewhere. Acceptance depends on the opinion of two referees and the decision of the Editorial Board. Papers accepted for publication are subject to editorial revision. Only original papers will be published and a copy of the Publishing Agreement will be sent to the authors of papers accepted for publication. Manuscripts will be processed only after receiving the signed copy of the agreement.

MANUSCRIPT STYLE

Manuscripts must be in English or Hungarian and clearly and concisely written. They should be typed double spaced with wide margins. Two copies of the manuscript should be submitted.

FORM OF MANUSCRIPT

Title. The title should be a clear and concise statement of the contents in not more than 14 words. A short running title of not more than 50 letters should also be supplied. This is followed by the authors' initials (full first name of women) and surname, and the name of the institution where the work was done. The mailing address of the authors must also be indicated here.

Abstract. This should not exceed 200 words and should outline briefly the purpose of the study and detail important findings and the authors' principal conclusions. Redundant phrases, generally known information and repetition should be avoided. Four or five keywords should also be supplied.

Introduction. This part should state briefly the nature and purpose of the work and cite recent important work by others.

Materials and methods. Describe materials, methods, apparatus, experimental procedure and statistical methods in sufficient detail to allow other authors to reproduce the results. This part may have subheadings. The experimental methods and treatments applied shall conform to the most recent guidelines on the humane treatment and care of animals.

Results. The experimental data should be represented clearly and concisely. Avoid repeating information presented in tables and figures.

Discussion should be focussed on the interpretation of experimental findings. Do not repeat literary data presented in the Introduction or information given in Results. References should be cited as follows: e.g. ... as observed by Faith and Trump (1976); or in parentheses: ... were found (Starr et al., 1978; Manson and Starr, 1979).

Acknowledgement of grants and technical help.

References. Cite only essential references. They should be arranged in alphabetical order of the authors' surnames, without serial numbers. The reference list at the end of the paper should contain

- for journals: names and initials of all authors, year of publication (in parentheses), colon, English title of the paper (if the original title is not English, indicate in parentheses, e.g. [in French]), journal title abbreviated according to the style used in Index Veterinarius, volume number, issue number in parentheses, first and last pages;
- for books: names and initials of authors/editors, year (in parentheses), title, publisher, place of publication, page number.

Tables. They should be typed on separate sheets and have a concise heading each. Tables are to be numbered sequentially by Roman numbers.

Figures. Good-quality glossy prints a half-tone illustrations and clear line drawings in Indian ink are accepted. Colour photographs will be accepted, but the extra cost of reproduction in colour must be borne by the authors (in 1995 US$ 260 per page). The number of the figure, the author's name and the top of the figure should be indicated lightly in soft pencil on the back. The figures are to be numbered sequentially by Arabic numbers. Captions should be provided on a separate sheet. All figures should be referred to in the text and their approximate place indicated on the margin.

Abbreviations and symbols. Quantities should be expressed in SI units. All abbreviations should be spelled out when first used in the text. Please identify unusual symbols in the margin.

Proofs and reprints. One set of proofs will be provided, which is requested to be returned within 48 hours of receipt to the Editor. Alterations in the text and especially in the illustrations are expensive and should be avoided. Fifty reprints of each paper are supplied free of charge. Additional reprints can be ordered at cost price at the time the page proof is returned.