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CONTENTS

Animal nutrition

Animal models in experimental atherosclerosis: A critical review. *Fekete, S.* 3
Nutritional factors affecting milk quality, with especial regard to milk protein: A review. *Hullár, I.* and *Brand, A.* 11
The influence of inedible raw material rendering method on meat meal quality. *Ristić, M., Kormanjoš, Š., Ćurčić, R.* and *Pupavac, Veselina* 33

Bacteriology

Treatment of calf pneumonia with tilmicosin. *Fodor, L., Varga, J., Gallowitsch, F., Horváth-Papp, I., Miklós, G., Lajcsák, A.* and *Harmath, A.* 41

Parasitology

Recent achievements in the chemotherapy of myxosporean infections of fish. *Molnár, K.* 51
Light and electron microscopic studies on the development of *Sphaerospora colomani* Baska, 1990 and *Chloromyxum inexpectatum* Baska, 1990. *Baska, F.* 59

Pathology

Mycoplasmal arthritis and meningitis in calves. *Stipkovits, L., Rády, Marietta* and *Glávits, R.* 73
Pathomorphological and immunohistological changes in lymph nodes of cows spontaneously infected by bovine leukaemia virus. *Levkut, M., Plank, L., Levkutova, M.* and *Konrád, V.* 89

Pathophysiology

Contribution to the pathobiochemistry of furazolidone-induced oxidative toxicity in chickens. *Sas, B.* 103

MAGYAR
TUDOMÁNYOS AKADÉMIA
KÖNYVTÁRA

Effects of concurrent administration of lead and selenium on some haematological and biochemical parameters of broiler chickens. *Khan, M. Z., Szarek, J., Krasnodębska-Depta, Anna* and *Koncicki, A.* 123
Biochemical changes of the milk in experimental caprine mastitis induced by *Mycoplasma* serogroup 11 (2-D). *Rana, J. S., Gupta, P. P.* and *Ahuja, S. P.* 139

Physiology

Clinical biochemical determinations in the Mangalarga-Paulista horse: Reference values. *Novelli, Ethel L. B., Rodrigues, N. L.* and *Chiacchio, S. B.* 151

Virology

Restriction endonuclease analysis of Hungarian bovine herpesvirus isolates from different clinical forms of IBR, IPV and encephalitis. *Magyar, G., Tanyi, J., Hornyák, Á.* and *Bartha, A.* 159
Serological survey for type D retroviruses and simian immunodeficiency virus in monkeys living in Hungary. *Gyuris, Ágnes, Vajda, G.* and *Földes, I.* 171
Detection of bovine herpesvirus 1 with various types of DNA probes. *Vilček, Š., Deliová, Iveta, Forgáč, O., Strojný, L., Takácsová, Ildikó, Harvan, M.* and *Benko, G.* 179

Announcement 191

Acta Veterinaria Hungarica 41 (1–2), pp. 3–9 (1993)

ANIMAL MODELS IN EXPERIMENTAL ATHEROSCLEROSIS: A CRITICAL REVIEW

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(Received September 16, 1992)

The most important features of lipoprotein metabolism are demonstrated and receptor-mediated endocytosis is emphasized. The different lipoprotein makeup of different animal species and humans is shown. The role of fibre, protein and polyunsaturated fatty acids in diet-induced hypercholesterolaemia is evaluated. The criteria of choosing the appropriate animal model for atherosclerosis research are discussed.

Key words: Lipoproteins, diet, fibre, protein, receptor-mediated endocytosis, rabbit, animal model, atherosclerosis, polyunsaturated fatty acids

Since the second half of the last century (Rokitansky, 1852; Virchow, 1856) there exists a remarkable agreement about the morphology of human atherosclerosis. The name derives from the Greek word "gruel". The need for a satisfactory animal model in experimental atherosclerosis arises from the fact that it is very difficult to study this multifactorial disease in man. It develops slowly and controlled studies on human subjects are very difficult to conduct. The *rabbit* is involved in the cholesterol topic in two ways: it is one of the best model animals for atherosclerosis research, and the rabbit carcass is one of the lowest cholesterol containing red meats (Lukefahr et al., 1989), even raised at a slow growth rate, using cheap feedstuffs.

About *lipoprotein metabolism in general.* Lipoproteins are protein–lipid complexes that carry triglyceride, cholesterol ester, cholesterol and phospholipid in the blood plasma. By ultracentrifugation, they can be separated into classes of different densities:

chylomicrons and very low density lipoproteins (VLDL), 1.006; low density lipoproteins (LDL), 1.066–1.062; and high density lipoproteins (HDL), 1.063–1.21 g/cm^3. Lipoproteins are spherical particles with a neutral lipid core (cholesterol ester and triglyceride) and an outer surface coat made up of lipoprotein, with association with cholesterol and phospholipid (Bartley, 1989). Their main function is to transport lipid through the blood. For review of the cholesterol transport see Norum et al. (1983).

Cholesterol is synthesized mainly in the liver and small intestine. Both tissues may export cholesterol as a constituent of triglyceride-rich lipoproteins (i.e. VLDL from the liver or chylomicrons from the intestine), or in the form of HDL. Partial triglycerid depletion of chylomicrons and VLDL leads to the formation of so-called "remnant particles" (or: intermediate density lipoproteins, IDL), that are metabolized primarily by the liver. IDL may be more completely hydrolysed to form LDL. The extent of remnant metabolism and LDL formation varies among species. LDL production is strong in humans; on the contrary, in rats IDL are metabolized by the liver and peripheral tissues and serve as a major cholesterol source for the steroidogenic tissues, e.g. the ovaries (Grummer and Caroll, 1988).

The amount and proportion of total blood cholesterol contributed by LDL and HDL vary among species. The LDL cholesterol accounts for the majority of the blood cholesterol pool in humans and pigs, whereas HDL cholesterol predominates in rats, (conventional) rabbits and cattle. The classical work of Nobel Laureates Goldstein and Brown demonstrated that human skin fibroblasts preferentially utilize LDL cholesterol for cellular functions, instead of *de novo* synthesized cholesterol. The LDL receptor is a glycoprotein that binds two proteins, apo B-100, which is the sole LDL protein in most species, and apo E, which is present in certain subclasses of HDL in some species. Lipoprotein binding initiates an adsorptive endocytosis (Csaba, 1990). In the acidic endosomes LDL particles dissociate from the receptor and the receptor is recycled to the cell surface. LDL will be hydrolysed in lysosomes into amino acids and cholesterol esters. Accumulation of LDL-derived cholesterol leads

to the suppression of the rate limiting enzyme of the *de novo* cholesterol synthesis (hydroxy-methylglutaryl coenzyme A reductase, HMG CoA reductase) and to the down-regulation of the LDL receptors (Brown et al., 1981).

The high density lipoprotein (HDL) uptake of tissues may take place by several mechanisms. HDL that contain apoprotein E are recognized by the LDL receptors and may be internalized by *receptor-mediated endocytosis,* as described previously. HDL that do not contain apoprotein E may deliver cholesterol to tissues by an alternative pathway, independent of the receptor-mediated endocytosis. Recently, it has been shown in cholesterol-fed rabbits that the impairment of VLDL and LDL catabolism is not limited to the receptor pathway either. This latter result makes the picture even more complicated. The WHHL rabbits – an inbred strain of rabbits lacking apo B, E-receptors – do not differ in this *receptor-independent LDL metabolism* from the conventional rabbits (Lacombe and Corraze, 1989).

Persons whose genetic makeup results in high levels of lipoproteins circulating in their blood have been categorized according to the lipoprotein pattern associated with the high plasma lipid level. Type II familial hypercholesterolaemia is characterized by an increase in LDL. Homozygous patients have 6–8 times higher cholesterol level than the normal and consistently develop heart disease by 20–30 years of age. Heterozygous persons (with one normal gene along with one for hypercholesterolaemia) also have predictable coronary heart disease (CHD) problems, but their cholesterol levels are only 2–3 times higher than normal. The formation of atheromas of persons with type II hypercholesterolaemia can be explained by a mutation (lack) of the LDL receptors. The produced LDL cannot be removed from the plasma owing to the lack of (liver) cell surface receptors for LDL (Church and Pond, 1989).

Although reference values for lipid metabolism in the rabbit resemble those of the rat, and the principal component is HDL, hypercholesterolaemia is more readily induced by dietary manipulations and it is the LDL fraction which increases. (This is in contrast to what is found for the rat, a species quite resistant to the induction of

hyperlipidaemia and atherosclerosis, except the Zucker Fatty Rat and RICO strains.) Even *the conventional rabbit* develops atherosclerosis very readily, so it has been widely used as a model to study this disease (Cheeke, 1987). Hypercholesterolaemia and atherosclerosis can easily be produced in the rabbit by cholesterol as well as casein feeding. Cholesterol- and casein-induced hypercholesterolaemia in rabbit develops in a similar manner; the cholesterol concentration increases first in the LDL fraction and subsequently in the VLDL fraction (Beynen et al., 1983).

The *Watanabe hereditable hyperlipaemic rabbit* (WHHL) is a very good model of familial hypercholesterolaemia (Watanabe et al., 1977). Homozygotes have mean serum cholesterol levels of 500 mg/dl, which may reach even 1000 mg/dl. *The principal fraction in these animals is low density lipoprotein cholesterol with only small quantities of HDL.* Affected animals develop atherosclerosis by 5 months of age (Loeb, 1989).

Hypercholesterolaemia and atherosclerosis in pigs are accompanied by coronary lesions. The mutant *pigs* have normal LDL receptor activity. Hypercholesterolaemia is due to abnormal catabolism of LDL in pigs with a mutant apo B. There is a defective binding of LDL to the normal receptor. The reduced catabolism specifically involves a more buoyant species of LDL (Bartley, 1989). Clarkson et al. (1988) proposed the pigeon and nonhuman primates as potential models for atherosclerosis. Radcliffe and Liebsch (1985) found the Japanese quail (strain SEA) to be a promising model for the study of atherosclerosis.

Some constituents of *dietary fibre* can reduce the blood cholesterol level. Fisher et al. (1967) as well as Borgman and Lightsey (1979) reported the hypocholesterolaemic effect of pectin, while Indira and Kurup (1989) that of neutral detergent fibre (NDF). The fibre reduces the blood cholesterol level in the following ways:

– by binding of bile and bile acids in the lumen of the intestine (West et al., 1989); and

– by regulating cholesterol metabolism through the released volatile fatty acids (Bergman, 1990). The latter means that propionate

inhibits HMG CoA synthetase activity in the tissues; the short-chain fatty acids, in general, tend to decrease HMG-CoA reductase, both indispensable in cholesterol synthesis.

The nature of *dietary protein* also affects cholesterol metabolism. For example the feeding of rabbits with a ration containing casein results in elevated serum cholesterol, whereas diets containing soybean protein maintain a low serum cholesterol level (Carroll and Hamilton, 1975). The cholesterolaemic effect of the above-mentioned proteins is attributed either to the lysine:arginine ratio (Kritchevsky, 1979) or to the influence exerted on the enterohepatic circulation of bile acids and cholesterol (Beynen et al., 1983). The type of fibre can erase or enhance the cholesterolaemic effect of dietary protein (Kritchevsky et al., 1977). *The rabbit is highly suitable for the investigation of the above-mentioned questions, because one can change the protein and fibre level between wide limits* (Fekete, 1989), possibly owing to adaptation by caecotrophy.

The discrepancy of opinions concerning the direct relationship between diet composition and coronary heart disease (CHD) can be resolved by the fact that the *fibrinolytic system* of the organism also plays an important role in the predisposition to, or prevention of, thrombosis and CHD. Since fibrinolysis may be influenced by a variety of nutritional factors (Ogston, 1985), which are very different according to feeding habit, social situation, geographical area, etc., the picture becomes more complicated. The findings of Tangney and Driskell (1981) suggest an enhancement of thrombin-induced phospholipase activity in platelets of vitamin E-deficient rabbits. There was a marked reduction in the percentage of aortic intimal surface covered by plaque, in aortic weight and cholesterol content in chromium-treated animals (Abraham et al., 1991). Low serum selenium levels and glutathione peroxidase (GSH-Px) activities have been associated with low serum levels of high density lipoprotein (HDL) cholesterol, and stimulation of GSH-Px improved the serum lipoprotein profile (Luoma et al., 1990). Lipid peroxidation and lipid accumulation occur in close association in the atherosclerotic plaque (Piotrowski et al., 1990).

High-linoleic-acid diets (and in general polyunsaturated fatty acids, PUFA) were associated with a drastic reduction in the incidence of coronary heart disease in humans, and serum cholesterol content as well as platelet aggregation were significantly reduced (Hornstra, 1982). The latter statements show the complexity of the question.

Clearly, much more information is required before a unifying hypothesis can be proposed for the diet–CHD interaction. On the other hand, this leaves a large area for research, in which the rabbit will have an important place. We can agree upon the choice of experimental model animal with Constantinides (1956) who stated in his classical work that "In view of recent developments, we are forced to answer that no single species is best, but we can learn different things from different species". Great caution should be used in extrapolating results obtained from the animal model to the human disease.

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NUTRITIONAL FACTORS AFFECTING MILK QUALITY, WITH ESPECIAL REGARD TO MILK PROTEIN: A REVIEW

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This paper reviews some of the recent scientific literature that relates nutritional management to protein concentration in the milk of dairy cows. Nutritional management of the dairy cattle was organized under the headings of nutrition and body condition before calving, forage to concentrate ratio, type and treatments of forage, dietary fibre, dietary protein, dietary amino acid supplementation, dietary fat, dietary carbohydrates, concentrate composition, vitamins, feed additives, and feeding frequency. The *level of feeding* and *rate of liveweight* change prior to calving within "normal" limits seems to have relatively little effect on milk yield and composition. Cows which are thin at calving, are biologically more efficient because they produce more milk directly from food rather than from mobilization of body reserves. Increasing the *forage content* of the diet leads to increased milk fat and depressed milk protein as well as milk yield, and vice versa. Modification of *forage type* and the treatments of forage seem to exert a relatively weak effect on milk composition. Increasing *neutral detergent fibre* (NDF) concentration of the diet from 25% to 37% on dry matter (DM) basis corresponds to a linear decrease in milk production, milk protein and a linear increase in the fat content of milk. When data were analysed by the source of roughage, a significant interaction of effects was found between NDF content of the diet on milk yield. Increasing the *protein concentration* of the diet up to 22% was found to raise milk yield and milk fat concentration, but an increase beyond that level had no consistent effect. A moderate amount of undegradable protein, however, can improve the milk yield and milk protein content of high-lactating cows. Milk protein concentration may be improved by various combinations of *ruminally protected methionine and lysine* if these amino acids are limiting in the diet. The inclusion of *free lipids* in diets generally increases the milk yield, but decreases milk protein concentration. *By-pass lipids* improve milk yield. Levels of dietary fat above 6–7 per cent lead to a depression of both total milk yields and fat and protein content. The source of *carbohydrate* in the concentrate has little effect on milk production when the concentrates are of similar metabolizable energy (ME) content. Dietary *beta carotene* has no significant effects on milk production or milk components, while *vitamin A*

may be more effective. *Niacin* supplementation may correct a milk protein depression induced by dietary oil. Increasing dietary *rumen-protected choline* increased the milk yield in a linear manner, although it had no consistent effect on milk fat or protein percentage. *Adding fat with Ca soaps of fatty acids*, which are inert in the rumen, increases both milk yield and milk fat. The addition of *sodium bicarbonate* to corn silage based diets partially alleviates milk fat depression. *Potassium carbonate* may act as a buffer which raises a depressed milk fat percentage. *Magnesium chelate* may increase milk protein percentage. Supplementation of diets with *magnesium oxide* in combination with *sodium bicarbonate, ammonium salts of volatile fatty acids, lasalocid,* either alone or in combination with sodium bicarbonate, seems to have no consistent beneficial effect on milk production or composition. Milk fat content or milk fat and milk protein content increase with higher *feeding frequency*; however, the effect is not consistent. It is suggested that dairy cows require not more than 8 hours/day access to feed if fed amounts required for *ad libitum* consumption at consistent times during the day, using corn silage based diets. Where intake is kept on a constant level, increased feeding frequency will not influence milk yield but could result in elevated milk fat percentage in cows fed fat-depressing diets.

Key words: Milk quality, milk protein, milk production, milk fat, dairy cows

The interest taken in the protein content of milk is increasing because a greater proportion of the milk supply is being processed into manufactured products and because of the change-over to component pricing systems. Most countries with a highly developed dairy industry pay producers on the basis of both total volume and compositional quality of the milk, and many have a milk quota system. To maximize profitability, a farmer must produce a specified amount of milk of optimum quality and composition.

Genetics, nutrition and management are the most important factors which can influence milk composition including the concentration of milk protein. Traditionally, the dairy cow has been bred for a high milk output and a high fat content in the milk. Dairy genetics recently have begun to lay more emphasis on milk protein content as a breeding value.

In contrast to genetics, nutrition allows relatively rapid changes to be made in the yield and composition of milk. In the past, the principal aim of nutritional advice on milk composition was to show how milk solids concentration could be increased. This mainly applied to increasing the concentration of milk fat. Recently, customers

demand a larger quantity of animal products low in fat. Therefore, animal nutritionists are improving the protein content of milk through manipulation of nutritional management. However, changes induced in milk protein concentration by dietary factors are smaller than those occurring in other milk components.

The purpose of this review is to present some of the nutritional factors influencing milk quality in the light of the recent scientific literature.

Nutrition and body condition before calving

Studies on the effect of nutrition and body condition before calving on milk production performance in dairy cows have yielded conflicting results. Severe underfeeding may cause cows to calve with a condition score 2 or less (overall scale 1–5), which will reduce milk yield, fat and protein content (Blowey, 1992). Better body condition may be associated with a higher milk yield (Land and Leaver, 1981), although milk fat depression is more severe in cows fed a high-energy prepartum diet (Jaquette et al., 1988; Holter et al., 1990). Other studies (Boisclair et al., 1986; Nocek et al., 1986; Garnsworthy and Topps, 1982) suggest that the feeding programme of the dry period or body condition at calving has no effect on postpartum milk yield and composition. The discrepancy between these results arises from the different period of observation because the effects exerted on milk quality may disappear after the peak of lactation.

The level of feeding and rate of liveweight change prior to calving within "normal" limits seem to have relatively little effect on milk yield and composition. However, most authors have found a negative relationship between body condition at calving and voluntary feed intake in early lactation. It is clear that body condition at calving can profoundly influence the way in which a cow adapts her metabolism to the onset of lactation (Reid et al., 1986). Results (Treachert et al., 1986; Garnsworthy and Jones, 1987) support the observation that cows which are thin at calving (condition score 2.5 on the overall scale of

1–5), are biologically more efficient because they produce more milk directly from food rather than from the mobilization of their body reserves. Cows that are fatter at calving (condition score 4.0) are characterized by a more expressed negative energy balance for a longer period than are cows thinner at calving.

Forage to concentrate ratio

The most pronounced effect arises from changing the forage to concentrate ratio. Elevated forage content of the diet leads to an increased milk fat and depressed milk protein level (DePeters and Smith, 1986; Grummer et al., 1987) as well as depressed milk yield (Rijpkema et al., 1990; Tessemann et al., 1991; Hansen et al., 1991; Llamas-Lamas and Combs, 1991), and vice versa.

Milk fat is synthesized from fatty acids, acetate and butyrate, which are the dominant products of the ruminal fermentation of forage and other feeds containing fibre. Large quantities of grain are fed to dairy cattle in early lactation to provide the energy required for a high-level milk production. The rapid digestion of grain dramatically decreases the pH of the rumen content, which can cause digestive disturbances and a depression of fibre digestion, resulting in lowered milk fat and depressed dry matter intake. Finding an optimal forage to concentrate ratio is also very important (NRC, 1988). Eastridge et al. (1988) obtained the best results with a forage to concentrate ratio of 53:47. The extent the depression caused by the above disturbance varies with the materials fed, but as a "rule of thumb", the forage to concentrate ratio should not be allowed to fall below 60:40.

Type and treatments of forage

The effects of modifying dietary forage on milk composition seem to be relatively small. Briceno et al. (1987) analysed data from 20 experiments (1,688 cow-periods) with Holstein cows in early to mid-

lactation that were fed either complete mixed diets or fixed forage to concentrate ratios. Roughages included sugarcane silage, cottonseed hulls, corn silage, and alfalfa hay. It appeared that the source of forage had no effect on milk yield and composition. Erdman (1988) examined the effects of forage pH on feed intake and found that a forage pH of 5.7 was optimum.

Many researchers have dealt with the role of forage particle size (Rogers et al., 1985a; Erdman, 1988; Belyea et al., 1989; Grant et al., 1990a; Grant et al., 1990b). Actual milk production was unaffected, but the results suggest that reduced particle size of forage produces metabolic changes which can be explained by the glycogenic theory for milk fat depression. The critical particle size is approximately 10 mm.

The digestibility of total dietary dry matter is higher when early-cut forages make up the forage component of the lactating cow ration instead of late-cut forages (Cleale and Bull, 1986; Nelson and Satter, 1990; Llamas-Lamas and Combs, 1990). Furthermore, the inclusion of early-cut forage, which is higher in energy than is late-cut forage, in the ration of early-lactating cows is associated with higher milk protein concentration (DePeters and Smith, 1986).

Some authors compared the effects exerted by different treatments of forage on milk yield and composition. Neither the chemical drying agents (consisting of potassium carbonate and sodium carbonate) of alfalfa (Oellermann et al., 1989; Ziemer et al., 1991) nor alkali and ammonia treatment (Canale et al., 1988; Kung et al., 1989) affected milk yield, milk fat or milk protein. Nor did the use of non-corrosive forage stabilizers (low-acid stabilizer) and microbial inoculation (Deetz et al., 1989; Kent et al., 1989) have an effect on milk yield and milk components.

Dietary fibre

Researchers have examined the potential of formulating dairy cattle diets on the basis of *Neutral Detergent Fibre (NDF)*, which

represents the slowly digestible cell wall fraction. NDF has been proposed as an alternative for assessing the total fibre content of feeds.

Increasing NDF concentration of the diet from 25% to 37% (on DM basis) results in linear decrease in milk production and milk protein, and a linear increase in the fat content of milk (Canale et al, 1990; Beauchemin, 1991).

The potential of using NDF to formulate dairy rations appears to be limited within roughage sources because no constant optimum value is available for maximum DM intake and milk yield among all roughage sources. For example, feeds of similar NDF content, such as cottonseed hulls (90% NDF) and sugarcane (87% NDF) have been shown to differ in bulk density (kg of DM/litre). When the data were analysed by the source of roughage, a significant interaction was found between NDF content of the diet and its effect on milk yield (Briceno et al., 1987).

Dietary protein

Dietary protein concentration generally exerts only minor influence on milk composition. However, as is the case with energy underfeeding, severe protein underfeeding tends to reduce milk protein concentration. Thus, the total percentage of crude protein (CP) in the ration can be a limiting factor to milk protein content (Orskov, 1982; Klusmeyer et al., 1990a; Small and Gordon, 1990). Increasing the protein concentration of the diet (Holter et al., 1985; Leonard and Block, 1988; Zimmermann et al., 1991) up to 22% was found to increase milk yield and milk fat concentration, but its elevation beyond that level had no consistent effect (Gordon, 1977; Grings et al., 1991). According to Kreuzer and Kirchgessner (1985), a higher intake of crude protein reduces N utilization for milk production. When cows are fed to minimize body fat mobilization, the response to increased dietary protein level is weak (Holter et al., 1985).

The protein requirements of high-producing dairy cows in early lactation are being revalued and must allow for the different degradabilities of feed protein. Microbial protein synthesis may provide only 60 to 80% of the amino N requirements of milk production (Guillaume et al., 1991); thus, high-producing cows must consume substantial amounts of protein that pass the reticulo-rumen undegraded. A high level of rumen degradable protein (RDP) during peak lactation can decrease milk yield and increase milk fat (Higginbotham et al., 1989a,b). For maximum milk production, high-lactating cows may need increased quantities of undegradable protein (UDP) in addition to microbial protein. A moderate amount of UDP can improve milk yield and milk protein content (Glenn et al., 1986; Small and Gordon, 1990; Winsryg et al., 1991).

In mid- and late lactation, the reduced metabolizable protein requirements for milk yield and higher feed intake relative to milk production allow rumen microbial protein to meet more of the metabolizable protein requirement with lower requirements for UDP (Robinson and Kennelly, 1988; Robinson et al., 1991).

Intra-abomasal infusions of sodium caseinate may increase milk and milk protein production and decrease milk fat percentage, although the results demonstrate that both ruminal degradability of dietary protein and the form of infused protein exerts an influence on the amino acid nutrition of lactating dairy cows (NRC, 1988; Seymour et al., 1990).

Dietary amino acid supplementation

Microbial protein has an important role in the pattern of amino acids entering the small intestine. The failure of microbial protein to supply amino acids in amounts sufficient for supporting high milk production has rendered it necessary to feed amino acids that escape ruminal degradation. Abomasal infusions of casein have been used to identify amino acids limiting to milk and milk protein production (Schwab et al., 1976; Rogers et al., 1979). Researchers suggested that

methionine may be the most limiting amino acid for milk protein synthesis (Shirley, 1986; NRC, 1988); however, direct evidence is limited. Amino acids supplied directly to the rumen are rapidly deaminated. Methods are therefore needed to protect amino acids from bacterial degradation. Feeding ruminally protected methionine (RPMet) diets may increase milk yield and milk protein content (Illg et al., 1987; Casper et al., 1987; Casper and Schingoethe, 1988, Schmidt, 1989, Cenkvári, 1990) because methionine is the first limiting amino acid in heat-treated soybean meal (HSBM). The addition of urea to diets containing HSBM may sufficiently increase ruminal lysine production so that the diet would then be limiting for methionine. In this case, added RPMet can also improve milk production (Munneke et al., 1991). According to Yang et al. (1986), feeding RPMet did not increase milk yield, milk fat, or milk protein. This lack of responses to RPMet indicated that methionine was not the first limiting amino acid in their diet.

Research with forms of methionine that have the potential to escape ruminal degradation has shown variable results. An increase in milk fat is the most common effect (Higginbotham et al., 1987; Hansen et al., 1991; Hegedűs and Brydl, 1992) but protein source in the basal diet (Donkin et al., 1989), forage type and forage to concentrate ratio also have an influence (Hansen et al., 1991).

In a multiple-site study, lactational performance of dairy cows fed diets with various combinations of RPMet and rumen protected lysine (RPLys) were compared with that of unsupplemented control cows. Milk protein was improved by various combinations of RPMet and RPLys when cows consumed a diet containing corn silage, corn gluten meal and urea (Rogers et al., 1987; Donkin et al., 1989), because Met and Lys are limited in these diets. Although plasma lysine and methionine are consistently increased in cows consuming diets supplemented with RPLys and RPMet, additional research is needed to define the optimal range of amino acid supplementation for milk protein. The effect of RPMet and RPLys supplementation may depend on the amount of added dietary fat. The addition of RPMet and RPLys increases total milk N in rations with high fat (3.9% added fat) but not

in high-concentrate diets (Chow et al., 1990). Results suggest that substitution of blended fat for concentrate leads to a reduction in microbial growth in the rumen, reducing the amino acid supply for protein synthesis. Providing additional amino acids postruminally may increase milk N even with diets containing fat.

Dietary fat

Feeding high-concentrate diets may result in excessive soluble carbohydrate intake, ruminal acidosis and milk fat depression. The inclusion of fat in dairy cattle rations can increase caloric density without reducing fibre content, thus increasing energy intake and the efficiency of energy utilization.

Lipid supplements have substantial and wide-ranging effects on both the yield of milk and the concentration of fat and protein. The inclusion of free lipids in diets generally increases milk yield but decreases milk protein concentration (Magdus et al., 1988; DePeters et al., 1989; Schingoethe and Casper, 1991). The cause of milk protein depression is poorly understood but is almost certainly extraruminal (Sutton and Morant, 1989). Smith et al. (1978) proposed that milk protein depression resulting from feeding high-fat diets to dairy cows might be due to a reduced availability of glucose, as glucose is a major substrate for the synthesis of both lactose and protein. Some studies suggest that high-fat diets in early lactation inhibit amino acid uptake by the mammary gland (Palmquist and Moser, 1981; Casper and Schingoethe, 1989; Wilks et al., 1991). According to Cant et al. (1991), dietary fat induced milk protein depression persisted during abomasal casein treatments, indicating a mechanism independent of the cow's amino acid status. In an experiment of Hoffman et al. (1991), there were no significant interactions between the intake of supplemental fat and undegraded protein on milk yield or composition. The results do not support the strategy of increasing the intake levels of undegraded protein when supplemental fat is fed. Addition of ruminally protected amino acids (Canale et al., 1990) or bovine somatotropin (Lough et al.,

1988) to fat-supplemented diets may help alleviate the milk protein depression found with added fat. At present there is no satisfactory explanation for the depression in milk protein concentration with lipid-supplemented diets.

The net effect of a lipid supplement on milk fat concentration depends on the balance of several different mechanisms (Sutton and Morant, 1989). Although feeding fat increases the energy density of the diet, it does not ensure that more net energy is available for milk production. This lack of an energy response occurs when fat supplementation causes one or several of the following events: fat can interfere with the digestion of other dietary nutrients, causing increased energy excretion; fat is poorly digested and absorbed; and the presence of fat may reduce the animal's voluntary feed intake (Jenkins and Jenny, 1989). The melting point of fatty acids is one of the characteristics that influence the extent of this problem. Fatty acids with low melting points inhibit ruminal microbial growth and fibre digestion. The inhibitory effects of fat on ruminal digestion may be alleviated by protecting the fat from microbial attack in the rumen. Rumen inert fat increased caloric density without compromising fibre, and increased energy intake for higher milk yield during early lactation when cows fail to consume sufficient feed (Lubis et al., 1990). By-pass lipids improve milk yield (Jenkins and Jenny, 1989; Eastridge and Firkins, 1991), but saturated fats such as tallow are used only to a maximum of 5–6 per cent to increase the fat content of milk. Milk protein content will, however, be depressed, but as milk yield is likely to increase, overall milk protein yield remains constant (Blowey, 1992). Levels of fat above 6–7 per cent lead to a depression of both total milk yield and fat as well as protein content.

Dietary carbohydrates

The readily available energy source is one of the most limiting factors for microbial growth and protein synthesis in the rumen of dairy cows. Structural carbohydrates have been used to predict the

energy value and intake of feeds, but the digestibility of this material may vary widely. Nonstructural carbohydrates such as starch are usually more digestible and often comprise a larger proportion of the carbohydrate.

Much of the research in this area has focussed on effects of carbohydrate source. De Visser et al. (1990) observed that milk protein content was higher for diets containing barley and corn, compared to ensiled beet pulp and ensiled maize bran. In their opinion, the lower protein content of milk when feeding beet pulp can be explained by a longer period of negative energy balance, while the lower milk protein in cows fed a diet of maize grain probably resulted from a reduced microbial protein synthesis. According to Wilks et al. (1991), both whole cottonseed and rice grain depressed milk protein percentage when substituted for concentrate. Another study concluded that the source of carbohydrate in the concentrate has little effect on milk production when the concentrates are of similar ME content (Sutton et al., 1987; Casper and Schingoethe, 1989).

Casper et al. (1990a) conducted an experiment to test the hypothesis that increasing amounts of fermentable carbohydrates in a high-fat diet may prevent the depression of milk protein content that usually occurs with high-fat diets. The concentration of milk fat and protein did not change but milk production did increase.

Infusion of exogenous glucose to dairy cows (Amaral et al., 1990) did not change milk production, milk fat and the composition of milk. Another study using both urea and starch also failed to demonstrate an increase in milk yield and milk fat. Milk protein decreased when additional starch was fed to cows (Cameron et al., 1991).

These studies used total mixed rations with a forage to concentrate ratio of 50:50. The effect of starchy concentrate at another ratio was discussed under the heading "Forage to concentrate ratio".

Concentrate composition

Numerous studies have investigated the effects of soybean or treated (roasted, extruded, popped) soybean meal and its involvement in dairy diets. Full-fat soybeans contain approximately 18% fat and 40% crude protein on a dry basis. It has been utilized in dairy rations to supply additional fat and protein during the first third of lactation. Although soybeans can be fed raw, appropriate heat processing increases the amount of protein that escapes ruminal degradation. The response of lactating dairy cows to inclusion of soybean or heat-treated soybean meal in diets has not been consistent. Milk protein percentages are higher when soybean meal is fed, compared to when extruded soybeans are fed (Schingoethe et al., 1988). Another study (Faldet and Satter, 1991) reported that feeding heat-treated soybeans resulted in higher milk and milk protein production as compared to raw soybeans. Yield increases were not always observed, because postruminal supply of the amino acids limiting for milk production may not have increased when proteins with low rumen degradability were fed. Also, positive responses were not always reported when extruded soybeans were fed, because cows may not have required more protein than that already supplied by the control protein diets (Annexstad et al., 1987). Voss et al. (1988) reported that addition of heat-treated soybeans had little or no effect on milk yield when the diet was based on corn silage. These authors suggested that heat-treated soybean may offer a greater potential for increasing milk yield when diets are based on alfalfa hay because of the greater solubility of its protein.

Roasted soybeans did not provide any additional benefit over raw soybeans (Bernard, 1990; Scott et al., 1991). There is scarce information regarding the optimum heat exposure for soybeans intended for ruminal diets. The additional heat treatment may reduce lysine availability by binding some lysine irreversibly to sugar, thereby causing lysine to become more limiting than methionine in those diets.

Data available on heat treatment of other oil seeds are limited. Added fat from sunflower seeds, coupled with the higher fibre of the seed hulls, might enable increased energy intake and efficiency without

reducing the fibre content of the ration. Higher fibre might help counteract the milk fat depression. Extruded sunflower seeds showed no benefits over extruded soybean meal (Drackley and Schingoethe, 1986), but according to the authors it is possible that this supplement would show more advantage if fed in a lesser amount.

Combinations of extruded soybeans and corn gluten meal were not advantageous compared with soybean meal as a supplemental protein source (Annexstad et al., 1987; Wohlt et al., 1991). Although cottonseed is low in lysine content, it is a reasonable source of methionine which is the limiting amino acid in soybean. Studies comparing diets with or without whole cottonseed have shown the following: no effect on actual milk yield (DePeters et al., 1985; Hein et al., 1990), decreased milk fat and milk protein percentage (DePeters et al., 1985), or similar percentages (Hein et al., 1990). Dietary free oil seeds depress milk protein concentration, which has been observed with whole cottonseed, unheated and heated soybeans and soybean oil (Mohamed et al., 1987).

Rapeseed fed at levels up to 14% of the diet did not affect milk yield, milk protein or milk fat percentage but did change milk fatty acid composition (Wiesen et al., 1990).

Using corn gluten in dairy rations may be advantageous because it contains on DM basis a comparable amount of NE_L but twice as much crude protein and five times as much NDF as does corn. It can replace 27% of dietary DM without altering milk yield and composition (Bernard et al., 1991). Feeding 20% corn gluten and 1% sodium bicarbonate was an effective replacement for NDF from corn silage (Firkins et al., 1991). Wet corn gluten feed is also an adequate supplement for dairy diets (Armentano and Dentine, 1988; Gunderson et al., 1988; Bernard et al., 1991).

Although wheat is widely used for livestock feeding, the amount that can be used in rations of dairy cows has not been defined well. Because the rate of ruminal degradation of both the carbohydrate and protein of wheat is relatively high, use of a large amount (above 40% of the concentrate) of it may decrease milk yield (Acedo et al., 1987).

Fish meal is a source of lysine, arginine and methionine in a form relatively resistant to microbial degradation. However, in some studies milk fat depression resulted (Blauwiekel et al.,1990; Wohlt et al., 1991). This effect may have been due to the high fat content of fish meal and the resultant deleterious effect on rumen microbial metabolism or on the utilization of fatty acids by the mammary gland (Blauwiekel et al., 1990).

Vitamins

Dietary beta carotene has no significant effects on milk production or milk components (Rakes et al., 1985). Vitamin A may be more effective: cows supplemented with 170,000 IU of vitamin A produced significantly more 4% fat corrected milk (FCM) (Oldham et al., 1991).

A consistent effect of most dietary fat sources on milk is an increase of milk yield and milk fat but a reduction of milk protein percentage. Using niacin in diets containing whole cottonseed may prevent milk protein depression (Horner et al., 1986). The protein supplement (heat-treated soybeans) by niacin interaction also suggests that niacin feeding corrects a dietary oil induced milk protein depression (Driver et al., 1990).

Changes in adipose and hepatic metabolism during early lactation suggest a possible role for lipotropic agents. Dietary choline is degraded rapidly in the rumen. A study with postruminal infusion of choline and methionine, with or without an inhibitor of methyl transfer for choline synthesis, suggested that methionine functions as a methyl donor for choline (Sharma and Erdman, 1988). Choline may be effective in increasing milk fat, possibly through its effect on hepatic lipid metabolism (Atkins et al., 1988). According to Erdman and Sharma (1991), increasing dietary rumen-protected choline causes a linear increase in milk yield although it has no consistent effect on milk fat or protein percentage. Data from this experiment suggest that choline may be a limiting nutrient for milk production.

Feed additives

Feeding large amounts of concentrates with small amounts of forage may necessitate to use mineral salts, often referred to as buffers, to prevent depressions in rumen pH and to improve feed intake, apparent nutrient digestibility, milk production, milk composition and animal health (Clark et al., 1989, Fekete et al., 1989). Adding fat with Ca soaps of fatty acid which are inert in the rumen, results in a higher milk and milk fat production, because Ca soaps can enhance the energy density of the ration and energy intake without lowering rumen cellulolytic bacterial activity (Schneider et al., 1988; Sklan et al., 1991).

Many researchers have demonstrated that addition of sodium bicarbonate to diets containing corn silage partially alleviates milk fat depression (Rogers et al., 1985b; Boisclair et al., 1986; Cassida et al., 1988; Canale and Stokes, 1988; Solorzano et al., 1989; Belibasakis and Triantos, 1991). Addition of zeolite to sodium bicarbonate had no significant effect (Johnson et al., 1988). Potassium carbonate may be also effective as a buffer, which raises a depressed milk fat percentage (West et al., 1987a). Cows fed diets containing potassium carbonate performed similarly to those fed sodium bicarbonate (West et al., 1987b).

Milk fat can be maintained at a normal concentration or elevated from a depressed concentration by adding magnesium oxide to high concentrate diets (Xin et al., 1989). Lough et al. (1990) could increase milk protein percentage with magnesium chelate. Using magnesium oxide in combination with sodium bicarbonate, no significant interactions were found in animal performance (Teh et al., 1985; Arambel et al., 1988).

Dietary cation-anion balance can be expressed quantitatively as milliequivalents of (Na + K) – Cl/100 g of dietary dry matter. This balance affects acid-base status in the blood and the performance of lactating dairy cows. Sodium chloride addition did not change milk yield or composition (Amaral et al., 1985). Results of studies using NaCl or KCl indicate that the balance of Na and K to Cl in the diet is a

more important determinant of dietary impact on acid-base status than is the actual dietary concentration of Na, K and Cl (Tucker and Hogue, 1990).

Supplementation of diets with ammonium salts of volatile fatty acids has no effect on the milk production and milk components of dairy cows (Peirce-Sander et al., 1985; Klusmeyer et al., 1987).

In contrast to early-lactation cows, cows in mid- to late lactation are generally in a positive energy balance. High-forage diets promote high-acetate ruminal fermentations, whereas ionophore antibiotics generally increase propionate. Therefore, cows in mid- to late lactation may respond positively to ionophores. The hypothesized changes of milk composition are expected to be accompanied by increased ruminal propionic acid, although the use of lasalocid (Johnson et al., 1988; Weiss and Amiet, 1990) did not affect milk production or milk composition.

Feeding frequency

Increased frequency of feeding stabilizes diurnal fluctuation of ruminal fermentation patterns. Milk fat percent (Nocek and Braud, 1985) or milk fat and milk protein percent (Yang and Varga, 1989) increased with higher feeding frequency, however the effect is not consistent (French et al., 1990; Klusmeyer et al., 1990b). There is a relative scarcity of works dealing with the eating time of and its effect on milk production. Milk yield and composition were unaffected by feed access time (Erdman et al., 1989). Results suggest that dairy cows require not more than 8 h/day feed access if fed *ad libitum* during the day using corn silage based diets. Where intake is kept constant, increased feeding frequency will not influence milk yield but could result in elevated milk fat percentage in animals fed fat-depressing diets (French and Kennelly, 1990).

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THE INFLUENCE OF INEDIBLE RAW MATERIAL RENDERING METHOD ON MEAT MEAL QUALITY

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The dependence of meat meal quality on inedible raw material rendering method was studied. The conventional batch dry rendering method with screw press defatting and the semi-continuous wet rendering method with centrifugal defatting were employed for processing inedible raw material (76.5% soft offals, 15% industrial bones and 8.5% swine cadavers). Amino acid destruction in the protein component was higher with the conventional batch dry rendering method. The contents and biological activities of lysine, methionine and cystine show that the nutritive value of meat meals produced by the conventional batch dry rendering method is lower than that of meat meals obtained by the semi-continuous wet rendering method.

Key words: Meat meal, rendering method, nutritive value, amino acid content

The quality of meat meals produced by rendering inedible raw by-products of the meat industry and dead animals depends on the composition of the raw material. Meat meals deficient in cystine, methionine and tryptophan are obtained from raw materials containing higher amounts of connective tissue (Black and Veiss, 1965; Fajviševskij and Liberman, 1984; Ristić and Kormanjoš, 1989). The quality of meat meals is greatly influenced also by the rendering process employed. Donoso et al. (1962), Skurray (1974), Ristić et al. (1983), Ristić et al. (1989) and Fajviševskij and Liberman (1984) have found that heating decreases protein digestibility and also causes the

loss of total and available lysine and essential amino acids containing sulphur. Similarly, Lea and Hanon (1950), Skurray (1974) and Ristić et al. (1983) have demonstrated losses in total and essential amino acids caused by heating.

The aim of this work was to study the influence of rendering process on meat meal quality.

Materials and methods

The conventional batch dry rendering method with screw press defatting and the semi-continuous wet rendering method with centrifugal defatting were compared.

Conventional batch dry rendering method. The precrushed raw material was indirectly heated up to a temperature of 135 °C in the cooker at a pressure of 0.275 MPa. For sterilization, the material was cooked for 18 min with continuous agitation. After sterilization the pressure was reduced to the atmospheric pressure and the temperature was lowered to 92 °C. The material was dried at that temperature for 120 min, then drying was continued to a water content of cca 6% at a temperature of 110 °C for 50 min. The dried material was discharged into the percolator for draining the free fat. The residue from the percolator, which still contained about 35% fat, was discharged into the defatting equipment. The defatted material was cooled and milled.

Semi-continuous wet rendering method. After the addition of process water (0.4 ton per 1 ton of raw material) the precrushed raw material was cooked and sterilized by indirect steam heating in the cooker. The cooking and sterilization period lasted 18 min at 135 °C and at a constant pressure of 0.275 MPa. The remaining content of the cooker was defatted in the decanter and centrifugal press. The defatted material was discharged to a continuous dryer in which it was held for about 20 min at 80 °C temperature until it reached a water content of 7%. The cooled material was milled to a granulation adequate for meat meal.

The raw materials used in these studies were equalized by structure and they consisted of 76.5% soft offals, 15% industrial bones and 8.5% dead swine.

The macrochemical composition and protein digestibility of the meat meals were determined by AOAC methods (1980). Amino acids were determined with a Biotronic-Model LC 5001 analyser. The samples were hydrolysed prior to analysis, and cystine and methionine were oxidized with performic acid. Tryptophan was determined spectrophotometrically. The biological activities of amino acids were calculated by the method of Akeson and Stahman (1964).

Results and discussion

The quality of meat meals and their value for animal feed is largely dependent on their chemical composition and the rendering method employed. The following parameters most affecting the quality of meat meals were studied: chemical composition, amino acid composition and digestibility, and biological value of certain important amino acids. The chemical composition of meat meals showed that, depending on the rendering method employed, meat meals of different quality were obtained (Table 1).

The meat meals produced had a varying fat content. The difference in fat content could mainly be attributed to the defatting method applied in the rendering processes studied. The decreased fat content obtained by the semi-continuous wet rendering process had no effect on the proportional increase of protein and mineral content. In the case of the conventional batch dry rendering process nearly the whole protein and mineral matter component was contained in the meat meals, while in the semi-continuous wet rendering process a part of this component was lost in the waste water, which is inevitable under large-scale conditions.

The main factors accounting for changes in the protein component are the temperature and the duration of processing. In

addition to these two factors, the medium containing the protein component is also very important.

Table 1

Values of proximate analysis of meat meals obtained by the rendering methods studied*

Item	Rendering method	
	Conventional batch dry rendering method	Semi-continuous wet rendering method
Moisture	5.24	6.61
Crude protein	57.13	57.65
Crude fibre	1.64	2.99
Crude fat	14.35	6.10
Ash	16.39	15.95
N-free extract	5.25	10.70

*averages of 10 samples analysed, in g/100 g of sample

These changes reduce the quality of meat meals only in the case of amino acid destruction or inactivation lowering protein digestibility and reducing the biological activity of amino acids.

Amino acids that were destroyed during the rendering process could not be detected in the proteins of the meat meals obtained. The inactivated amino acids could be detected and their value was determined on the basis of protein digestibility and biological activity of amino acids.

The content of individual amino acids and the biological activity of lysine, cystine and methionine are shown in Table 2.

As compared to meat meal produced by the conventional batch dry rendering method, meat meal produced by the semi-continuous wet rendering method contained a higher concentration of essential amino acids (36.42% vs. 42.38%).

Table 2

Amino acid composition and biological activity*

Amino acids	Rendering method			
	Conventional batch dry rendering method		Semi-continuous wet rendering method	
	g/100 g crude protein	Biological activity, % of total content	g/100 g crude protein	Biological activity, % of total content
Lysine	3.94	68.5	6.22	78.1
Histidine	2.26		2.34	
Arginine	6.03		6.86	
Threonine	2.67		3.23	
Valine	4.94		5.21	
Methionine	1.04	78.7	1.39	86.9
Isoleucine	2.72		3.00	
Leucine	8.21		8.59	
Phenylalanine	3.82		4.44	
Tryptophan	0.79		1.10	
Cystine	0.71	50.3	1.14	59.6
Tyrosine	2.40		2.52	
Total amino acid content	92.17		99.23	

*averages of 10 samples

The differences in essential amino acid and total amino acid content resulted from the composition of raw materials (although it had been equalized by structure) and from the influence of temperature. The significance of temperature was especially expressed in the conventional batch dry rendering method in the phase of drying and pressing. Lysine activity in proteins of meat meal produced by the semi-continuous wet rendering method was significantly higher as compared to proteins in meat meals obtained by conventional batch dry rendering method (78.1% vs 68.5%).

Table 3

Protein digestibility in meat meals*

	Rendering method	
	Conventional batch dry rendering method	Semi-continuous wet rendering method
Digestible protein, in % of sample	49.34	54.48
Digestible protein, in % of crude protein	86.36	94.50

*averages of 10 samples

The content and activity of amino acids containing sulphur (cystine and methionine) in the proteins of meat meals obtained are indicative of oxidation changes occurring in the rendering processes studied.

The lower content of oxyamino acids (threonine and serine) found in the conventional batch dry rendering method shows that dehydration reactions were more expressed.

In the case of the semi-continuous wet rendering method *in vitro* protein digestibility was higher by 9.42% (Table 3).

The results of this study show that, in spite of some disadvantages mentioned here, both rendering methods produce meat meals of adequate quality for use in animal feeding.

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TREATMENT OF CALF PNEUMONIA WITH TILMICOSIN

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The therapeutic effect of a single injection of tilmicosin (10 mg/kg body mass) against natural cases of pneumonia of calves, compared to other conventionally used antibiotics, was examined under field conditions. The trials were conducted in three cattle herds and included the treatment of 135 calves with 82 controls. The animals' age ranged from 1 week to 4 months. Prior to the treatment and in case of death bacteriological examinations from nasal swabs and from the lungs were carried out. Clinical signs were recorded in all groups and in one group body mass gains were also recorded. After tilmicosin treatment, the body temperature returned to the normal level within 24 h and the animals recovered within 1 to 2 days. Ten calves (7.41%) showed relapse during the 3rd and 4th week after treatment in contrast to 9 (10.98%) in the control group. Three calves (2.22%) died in the groups treated with tilmicosin and 6 calves (7.32%) among the controls. In one of the treated groups the body mass gain was 6.2% higher compared to the controls at the age of 3 months. These results suggest that treatment with a single dose of tilmicosin is effective in most cases of calf pneumonia caused by bacteria.

Key words: Tilmicosin, calf, pneumonia, cattle

Pneumonia in calves causing considerable losses is a current issue in cattle breeding especially where large herds have been concentrated (McKercher, 1978; Merrill and Thonkinson, 1989; Yates, 1982). The aetiology of respiratory diseases in cattle is complex. It comprises stress and management faults together with infective agents including several bacterial species which can cause severe lung lesions

and, finally, death (Carter, 1973; Frank et al., 1987; Hoerlein, 1973; Yates, 1982). *Pasteurella haemolytica, P. multocida* and *Haemophilus somnus* are the bacteria most frequently isolated from cattle pneumonia in Hungary and in other countries as well (Fodor et al., 1988; Forray et al., 1984; Yates, 1982).

A new macrolide antibiotic, 20-deoxo-20-(3,5-dimethylpiperidine -L-YL)desmycosin (tilmicosin) has recently been developed for the treatment of respiratory diseases of cattle. *In vitro* tests proved the efficacy of tilmicosin against Gram-positive bacteria, *Pasteurella* and *Mycoplasma* species (Debono et al., 1989; Ose, 1987).

Colonization of the lungs by *P. haemolytica* was prevented and that by *Mycoplasma bovis* was considerably reduced if gnotobiotic calves were treated with tilmicosin before infection (Gourlay et al., 1989). A single subcutaneous injection of tilmicosin had a beneficial effect on the recovery of neonatal calves suffering from pneumonia (Ose and Thonkinson, 1988). One tilmicosin injection on arrival at the feedlot was capable of reducing the treatment rate in cattle during the first five days and during the first month; furthermore, the medicated group had improved average daily body mass gain and feed efficiency (Schumann et al., 1990). Compared to other commercially available antibiotics, tilmicosin proved to be superior as far as body temperature reduction, number of treatment days and the relapse rate were concerned (Merrill and Thonkinson, 1989).

Data on the efficacy of tilmicosin were limited and were from countries with different conditions, management and animal husbandry. Therefore it seemed to be worthwhile to examine the therapeutic effect of a single injection of tilmicosin against naturally occurring calf pneumonia in Hungary under field conditions.

Materials and methods

Farms, animals. The experiments were conducted in three herds of Holstein-Friesian or Holstein-Friesian x Hungarian Fleckvieh cattle. One farm had about 1,000 cows (farm A), one about 1,200 (farm B)

while on the third one 700 cows were kept (farm C). Calves showing symptoms of pneumonia were randomly assigned to two groups, one of which was treated with a single injection (10 mg/kg body mass of tilmicosin (Micotil®, Eli Lilly), while the other with conventionally used antibiotics according to the manufacturer's instructions. The calves' age was between 1 week and 4 months, on the average they were 1 month old. The number of animals in the experimental and in the control groups together with the antibiotics used for treating the control calves are summarized in Table 1. The management and the feeding of the two groups were the same. Their feed was not medicated.

Bacteriological examinations. Nasal swab samples taken from 7–15 pneumonic calves on each farm and organ (lung, liver, spleen, kidney) samples from 4 calves which had died were inoculated onto ox blood agar and cooked blood agar plates containing 0.5% yeast extract. The blood agar plates were incubated for 72 h in air at 37 °C, the cooked blood agar plates were incubated for the same time at the same temperature in an atmosphere containing 10% CO_2. The plates were examined daily, the isolated bacteria were identified according to standard methods (Cowan, 1974) and the serotypes of the *P. haemolytica* and the *P. multocida* strains were determined as described by Biberstein (1978) and Carter (1984).

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of tilmicosin for the isolated bacteria were determined by the tube dilution method in phenol red dextrose broth (Difco) and subsequent inoculations onto the above-mentioned ox blood agar plates. *Mycoplasma* spp. were examined as described elsewhere (Ernø and Stipkovits, 1973; Stipkovits et al., 1987).

Clinical and postmortem examinations. The calves included in the trial were examined daily till recovery or for 28 days. Clinical signs including respiratory rate, body temperature, appetite, behaviour and diarrhoea, together with nasal discharge and coughing (data not presented) were recorded and scored on days 0 to 10 and day 28 as follows:

Respiratory rate: 0: normal (15–40/min); 1: elevated (40–50/min); 2: polypnoe (>50/min).

Body temperature: 0: <39.9 °C; 1: 40.0–40.5 °C; 2: >40.5 °C.

Appetite: 0: normal; 1: reduced; 2: anorexia.

Behaviour: 0: normal; 1: dullness; 2: recumbency.

Lung lesions of the necropsied animals were also scored: 0: no lesion; 1: <5% involvement; 2: 6–20% involvement; 3: >20% involvement.

Thirty-two calves on farm A were weighed at the beginning of treatment and at the age of 3 months.

Table 1

Experimental design

Farm	Number of calves in		Antibiotics used for the treatment of control animals (number of calves treated)
	experimental group	control group	
A	50	50	Terramycin, Pfizer (30); Excenel, Upjohn (16); Tiamulin, HAGE (2); Tylan, Elanco (1); Gentamycin, Sanofi (1)
B	42	32	Terramycin, Pfizer (16); Tardomyocel, Bayer (5); Gallimycin, Sanofi (1); Sumetrolim, Egis (1); Vesulong, Ciba-Geigy (1); combination of Tetran, Chinoin, Lincospectin, Upjohn and Promptcillin, Biogal (8)
C	43	0	–

Data analysis. Each animal was considered as an experimental unit. The main parameters for evaluating the efficacy of treatment were mortality, body temperature, severity of clinical signs, duration of illness, and relapse rate. Data collected during the experiments were analysed by statistical methods. Mean values, standard deviation and significance were calculated (Sváb, 1973).

Results

Bacteriological examinations. The results of bacteriological examinations performed before the treatment are presented in Table 2, together with the MIC and MBC values obtained for the isolated bacteria.

Table 2

Bacteriological examinations before the experiment

Farms	Number of		Isolated bacteria	MIC*	MBC**
	positive swabs	swabs examined			
A	8	9	*P. multocida* A	5 (4)	10 (3), 5 (1)
B	3	10	*P. multocida* A	5 (3)	5 (3)
	5	5	*Mycoplasma* spp.	0.125 (5)	0.125 (5)
C	6	7	*P. multocida*	5 (2)	5 (2)

*MIC (minimum inhibitory concentration) and **MBC (minimum bactericidal concentration), μg/ml (number of strains examined)

Farm experiments. The data obtained in the farm experiments are summarized in Table 3. On farm A, tilmicosin treatment proved to be very effective and led to fast recovery, although scores in the tilmicosin-treated group for some symptoms were significantly higher than in the control group. No local reaction to tilmicosin injection was observed. Four relapses (8.0%) occurred in both groups. Two treated

(4.0%) and 4 control calves (8.0%) died of pneumonia in the experimental groups, out of which 3 (1 tilmicosin-treated and 2 controls) were sent for bacteriological examination. A tilmicosin-sensitive strain of *P. haemolytica*, serotype A1 was isolated in pure culture from the animal of the tilmicosin-treated group, while from the lungs of one control calf *P. multocida* and streptococci were cultured. From the third animal (control group) no bacteria could be isolated. The body mass gain of the treated animals at the age of 3 months was 6.2% higher than that of the controls but the difference was not significant.

Table 3

Average scores after tilmicosin treatment

		Farm A		Farm B		Farm C
		E	C	E	C	E
Number of animals		50	50	42	32	43
Death due to pneumonia		2	4	0	2	1
Number of treatments		1	2.13	1.57	2.25	1.12
Respiratory rate	day 0	1.71	1.33	1.57	1.16[b]	1.00
	day 10	0.08	0.41	0.50	0.45	0
	day 28	0.04	0.11	0.23	0.17	0
Body temperature	day 0	1.40	1.11[b]	0.83	0.47[c]	0.98
	day 10	0.13	0.24	0.19	0.10	0
	day 28	0.02	0.04	0.08	0	0
Appetite	day 0	1.69	1.17[a]	0.71	0.78	1.19
	day 10	0.19	0.59[a]	0.10	0.10	0
	day 28	0.08	0.54[a]	0.05	0.03	0
Behaviour	day 0	1.35	0.96[a]	0.12	0.06	0.33
	day 10	0.04	0.17[c]	0.05	0.03	0
	day 28	0.13	0.04	0.03	0	0
Lung lesions (average)		3	3	0	2	3

Significant difference at [a]$p<0.001$; [b]$p<0.01$; [c]$p<0.05$; E = tilmicosin-treated group; C = control group

On farm B, the treatment proved to be as effective as on farm A, but 6 (14.28%) relapses were observed, generally during the 3rd or 4th week after tilmicosin treatment. Five control animals (15.62%) showed a relapse. A local swelling the size of a nut appeared in 19 calves after administration of the drug. In some animals the swelling was slightly painful but disappeared before the end of the observation period. Only 2 control calves died of pneumonia on farm B, while no deaths occurred among the tilmicosin-treated animals.

On farm C only one treated calf died but in that case treatment was started very late. From the lungs of that calf *P. haemolytica* A1 was isolated in pure culture. The injection site showed some swelling in 2 calves but returned to normal within a few days.

Discussion

According to our results, tilmicosin injection proved to be effective for the treatment of calf pneumonia in the field.

Low concentrations of tilmicosin showed very good antibacterial effect against *P. multocida*, *P. haemolytica* and *Mycoplasma* spp. which are commonly isolated from calf pneumonia in Hungary. The favourable results of this experiment can be explained by the fast peak of tilmicosin in the plasma and the high and prolonged concentration of the drug in the lungs (Gourlay et al., 1989). The therapeutic effect of tilmicosin reached or surpassed that of other antibiotics used in the practice.

In most cases a single injection of tilmicosin was effective in treating pneumonia: body temperature dropped within a day, the animals recovered very quickly and the relapse rate was very low. Most relapses occurred in the 3rd or 4th week after treatment when the concentration of tilmicosin had decreased and it could not prevent colonization of the lungs by bacteria as a result of a reinfection. The number of calves which died was lower in the treated groups. Sensitivity testing of the strains isolated from treated animals which died suggested that there was no lack of sensitivity of the causal

bacteria, and indicated that the death of those animals could be attributed to the late initiation of treatment.

Our findings are in complete agreement with the results of Schumann et al. (1990) who administered tilmicosin to calves on arrival at the feedlot, and support the conclusions of Gourlay et al. (1989) and Ose and Thonkinson (1988). Our results on the good efficacy of tilmicosin correspond to data of other authors (Guichon and Jim, 1990; Laven and Andrews, 1991; Picavet et al., 1991) as well. According to the results discussed above, tilmicosin is an effective antibacterial agent against bacteria causing pneumonia in calves, and a single injection can be used with high success for the treatment of calf pneumonia in the field.

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RECENT ACHIEVEMENTS IN THE CHEMOTHERAPY OF MYXOSPOREAN INFECTIONS OF FISH

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Until quite recently, the therapy of fish diseases caused by myxosporeans has been an unsolved problem. Fumagillin is the only effective drug which can be successfully applied against several myxosporeoses in the form of prolonged feeding. It has been used with good results against sphaerosporosis of common carp, hoferellosis of goldfish, myxidiosis of eel, and proliferative kidney disease, whirling disease and ceratomyxosis of salmonids. Already a 4- to 6-day feeding of medicated diets containing 0.05–0.1% fumagillin was effective against the early blood stages of Sphaerospora, while toltrazuril failed to kill these parasites.

Key words: Chemotherapy, fish parasites, Myxosporea, fumagillin

The therapy of fish diseases caused by myxosporeans has long been an unsolved problem of ichthyoparasitology. To this very day, no really effective and easy-to-use drug has been available against these infections. Although Scolary reported the partial efficacy of the arsenic-containing acetarsol (Stovarsol) against *Myxosoma cerebralis* infection already in 1954, subsequent studies have failed to confirm his results. Taylor et al. (1973) described that furazolidone, mixed in the food of fish at a dose of 152–194 mg/kg, was effective against the above parasite. However, their results have not been confirmed either. Alderman (1986) tested the above two drugs and found them in-effective. At the same time, he found that two other antiprotozoic compounds, Clamoxiquin and Proquanil, reduced the intensity of *M. cerebralis* infection (whirling disease) when fed to rainbow trout.

If we accept that proliferative kidney disease (PKD) is caused by sphaerospores (an assumption which seems to be consistently supported by recent data), malachite green must also be counted among the drugs potentially effective against myxosporeans. Clifton-Hadley and Alderman (1987) reported that malachite green exerted a beneficial effect on the course of PKD and reduced its severity. Other authors including Ibarra et al. (1990) failed to see such an effect. Nor could we observe any efficacy of malachite green against myxosporean infections. The favourable effect observed by Clifton-Hadley and Alderman (1987) in the case of PKD may have rather been due to the action exerted by malachite green on ecto-parasitic protozoa and fungi, as that drug is highly suitable for eliminating various agents causing concomitant infections.

At present, fumagillin and toltrazuril are the only drugs which show promise in the control of diseases caused by myxosporeans.

The parasiticidal effect exerted by toltrazuril on myxosporeans has been reported by Mehlhorn et al. (1988) and Schmahl et al. (1989), who have found it effective against a *Myxobolus* and a *Henneguya* species. Other authors, among others Yokoyama et al. (1990), failed to observe such an anti-myxosporean efficacy. Similarly, our experiments with toltrazuril against *Thelohanellus* and *Myxobolus* species have yielded negative results (unpublished).

The antibiotic fumagillin has long been used for the control of diseases caused by different microsporeans. It was first used by Katznelson and Jamieson (1952) against nosema disease of honeybees. Kano and Fukui (1982) used fumagillin in fish for controlling the so-called BEKO disease, a disease of Japanese eel caused by microsporeans. These results prompted our team (Molnár et al., 1987) to conduct an experiment in which swimbladder in-flammation of common carp, caused by developmental stages of *Sphaerospora renicola*, was successfully treated with fumagillin. In that experiment, common carp were experimentally infected with late *Sphaerospora* blood stages (K stages) derived from common carp affected with swimbladder inflammation. By adding fumagillin to the feed at a rate of 0.1%, the development of sphaerosporosis, which took place in

100% of the control fish, was successfully prevented in the fumagillin-fed animals. Full efficacy was achieved after a 5-day feeding period. Voronin and Chernisheva (1991) tried out fumagillin in naturally infected common carp. By permanent feeding of the drug for 3–5 weeks, they successfully prevented the development of swim-bladder inflammation in intensive, tempered-water systems in which the disease occurred regularly, every year, and it developed among the control fish even in the year of the experiment.

Hedrick et al. (1988), who used fumagillin to control PKD, also reported a high efficacy. After feeding fumagillin for 7 weeks at a dose of 0.5–1 g/kg^{-1} fish body mass, neither parasites nor signs of PKD were demonstrable among experimentally infected chinook salmons. However, the above authors also pointed out that six weeks after the start of medication the fish given the higher dose of fumagillin lost their appetite. Later on, Lauren et al. (1989) reported that fumagillin fed for a prolonged period or at high doses caused signs of toxicity among rainbow trout.

By the continuous feeding of fumagillin in the diet, Székely et al. (1988) successfully prevented the development of *Myxidium giardi* cysts in elvers. However, they also mentioned that in their experiments the drug proved to be much more parasitostatic than parasiticidal, and some more developed cysts continued to develop after medication had been stopped. For this experiment, we must know that the glass eels, allegedly free of infection at the time of purchase, are in fact always infected latently and harbour more or less developed plasmodia in their renal parenchyma.

A similarly good efficacy was reported by Yokoyama et al. (1990) who used fumagillin against *Hoferellus carassii* infection causing kidney enlargement disease in goldfish. They also mentioned that fumagillin had been first used against myxosporeans by a Japanese researcher, Murakami (1980) who successfully treated a Myxobolus-induced disease of a marine fish species with it.

ElMatbouli and Hoffmann (1991) also obtained favourable results with fumagillin in whirling disease of rainbow trout, and observed no side effects during the 160-day period of medication.

Ching (unpublished) found fumagillin to be highly effective against experimentally induced *Ceratomyxa shasta* infection of chinook salmon. In the fumagillin-treated group no deaths occurred, while a substantial mortality was observed among the controls. At the same time, Ibarra et al. (1990), who used fumagillin against the same disease in rainbow trout, obtained very moderate efficacy. A similarly poor efficacy was obtained by Sitja-Bobadilla and Alvarez-Pellitero (1989) for fumagillin used against *Sphaerospora testicularis* infection of naturally infected sea bass.

These contradictory results may obviously be explained by the mechanism of action of fumagillin, which proved effective only against the presporogonic developmental stages. The swimbladder stages of sphaerospores and the PKX organism can be surely destroyed by fumagillin feeding; however, it is less effective against the more advanced sporogonic developmental stages, first of all those of species developing in cysts, and in these cases only a parasitostatic mechanism asserts itself. Thus, the efficacy of fumagillin treatment depends primarily on correct diagnosis and timed therapy of the presporogonic stage in the case of a given myxosporean disease of the fish. As this is difficult to carry out in the practice, prolonged fumagillin feeding would be needed, similar to what proved successful in the case of anticoccidials. However, Wishkovsky et al. (1990) reported that rainbow trout fed the therapeutic dose of fumagillin lost their appetite after two weeks and anorexia as well as mortality occurred after four weeks of medication. Sövényi (1992), who studied the effect of fumagillin feeding on the haematopoietic organs, reported weight loss of those organs and adverse changes in the composition of leukocytes present in the kidney. In spite of these changes, no mortality occurred among common carp tested in aquaria during the eight-week medication period (author's unpublished data).

From the above facts it is obvious that the treatment of fish diseases caused by myxosporeans requires further research. It must by all means be elucidated whether toltrazuril has any effect against myxosporeans. It must also be determined that against which diseases, in what form and at what dose can fumagillin, a compound which is

undoubtedly effective, be used. In order to answer some of these questions, I conducted experiments with toltrazuril and fumagillin in common carp infected with *Sphaerospora renicola* blood stages. The C blood stages chosen as a test model partly constitute the earliest development stage of different *Sphaerospora* species (Csaba et al., 1984; Molnár, 1988), partly – according to the observations of Odening et al. (1989), Voronin and Chernisheva (1991) and my own unpublished results – they correspond to a "late" parasite stage occasionally persisting in the blood for several months and causing a recurrence of infection at a later time.

The experiments were conducted in 40-litre aerated aquaria. Common carp fry weighing 10–15 g were used which had recovered from swimbladder inflammation and subsequent renal sphaerosporosis, and the C protozoa found in them corresponded to surviving stages that possibly caused recurrence of infection. In the fumagillin experiment, the experimental common carp were fed 0.1 or 0.05% fumagillin mixed to the common carp diet fed at 2% of the carps' body mass as indicated in Table 1. Toltrazuril was used as described by Mehlhorn et al. (1988) in the form of a single 4-hour or 6-hour exposure (Table 2). Infection of the fish by Sphaerospora blood stages was checked by microscopic examination of fresh preparations made from the choroid of the eye as recommended by Sövényi and Molnár (1990).

As indicated by the data shown in Table 1, 4- or 6-day feeding of a medicated diet containing 0.1 or 0.05% fumagillin resulted in a high degree of parasite elimination as compared to the controls, and no C blood stages were found in the group fed fumagillin for 22 days at the end of the trial. The four fish that remained infected after fumagillin feeding of shorter duration must have belonged to those weaker individuals which could not take up the diet in the necessary therapeutic dose.

According to data shown in Table 2, short exposures to toltrazuril solution remained ineffective, and no difference was demonstrable between the toltrazuril-treated groups and the control fish. It

can be stated that this very simple treatment procedure is not suitable for destroying Sphaerospora blood stages.

Table 1

Medication of common carp infected by C blood stages of *Sphaerospora renicola* by feeding a diet containing different concentrations of fumagillin

No. of experiment	Mode of application	Dose	Duration of treatment (days)	Day of examination after start of treatment	Number of fish		
					total	infected	negative
1	feeding	0.1%	4	6	10	1	9
2	feeding	0.05%	4	6	10	2	8
3	feeding	0.1%	6	8	22	1	21
4	feeding	0.05%	6	8	21	0	21
5	feeding	control		6	30	24	3
6	feeding	0.1%	22	25	15	0	15
7	feeding	control	25	25	15	10	5

Table 2

Exposure of common carp infected by C blood stages of *Sphaerospora renicola* to toltrazuril solution

No. of exp.	Mode of application	Dose	Duration of treatment (h)	Day of examination after start of treatment	Number of fish			
					tested	infected	negative	died after exposure
1	bathing	2 mg/l	6	7	10	6 (33%)	7	1
2	bathing	10 mg/l	4	7	10	2 (25%)	6	2
3	control	tap-water		7	10	5 (55%)	4	1
4	bathing	2 mg/l	6	5	15	9 (64%)	5	1
5	bathing	10 mg/l	4	5	15	9 (75%)	3	3
6	control	tap-water		5	15	9 (33%)	6	1

It seems that today only one drug, fumagillin, is suitable for controlling diseases caused by myxosporeans. As demonstrated by the experiments outlined above, already a short-term feeding of fumagillin-containing diet may effectively destroy the presporogonic stages. This was proved by Voronin and Chernisheva (1991) also in the practice. At the same time, against other myxosporean infections fumagillin can be used effectively only if the development and dynamic appearance of the given parasite is known. Only in that way can the optimum time of treatment be chosen, which is necessary for preventing the adverse side effects of medication by shortening the duration of feeding.

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LIGHT AND ELECTRON MICROSCOPIC STUDIES ON THE DEVELOPMENT OF *Sphaerospora colomani* BASKA, 1990 AND *Chloromyxum inexpectatum* BASKA, 1990

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During a parasitological survey conducted on sterlets (*Acipenser ruthenus* L.), the developmental stages of two new coelozoic species of *Myxosporea* were demonstrated in the kidney and in the urinary passages. *Sphaerospora colomani* develops vegetative stages in the interstitial capillaries of the kidney in winter. The ultrastructure of a large primary cell containing secondary, tertiary and quaternary cells was examined. The vegetative developmental forms were located beside the tubule in the cytoplasm of the endothelial cell. The primary cell developed in the cytoplasm of the endothelial cell, making the cell greatly enlarged. Young, developing and fully vacuolated secondary cells were seen in the cytoplasm. Two tertiary cells developed in each vacuole that contained free nuclei and quaternary cells. Each developmental stage of the parasite bore processes to increase the surface. The pseudoplasmodia of *Chloromyxum inexpectatum* were found mainly in the lower passages and, less often, in the renal tubules of all fish specimens examined. The vegetative stages were located among the capillaries of the Bowman's capsules. The structure of the Bowman's capsule had changed due to the xenoma developing inside. A thin, shaded area represented the demarcation zone between the xenoma and the host cells. The cytoplasm of the xenoma formed by host and parasitic cells contained nuclei and young, differentiated secondary cells. In the more developed secondary cells, generally two tertiary cells developed and, later on, a quaternary cell appeared in each.

Key words: Sterlet, *Myxosporea, Sphaerospora colomani*, *Chloromyxum inexpectatum,* development, light and electron microscopy

Earlier, before the dam at the Iron Gates was completed, several acipenserid species had inhabited the rivers of Hungary. Of the numerous species fished for earlier, it is the sterlet which has become

best adapted to freshwater, can be caught in large numbers in our rivers, and has a population increasing from year to year. The sterlet has been successfully propagated in Hungary, its feeding and management technology has been elaborated, and attempts to hybridize it with other acipenserid species have also succeeded. This is how the parasitological examination of sterlets from natural waters has gained importance, so that the potential hazard of animal hygienic problems posed by parasites introduced to intensive systems could be predicted and the elaboration of an animal health technology could be started with knowledge of the parasitoses affecting the broodstock.

The results of the survey comprising an about six-year faunistic work are contained in Table 1.

Table 1

Species identified during the parasitological survey of Hungarian sterlets

Trichodina spp.	*Amphilina foliacea*
Cryptobia acipenseris	*Diplostomum spathaceum*
Haemogregarina acipenseris	*Skrjabinopsolus semiarmatus*
Goussia vargai	*Acrolichanus auriculatum*
Goussia acipenseris	*Nicolla skrjabini*
Myxidium sturionis	*Hysterothylacium bidentatum*
Chloromyxum inexpectatum	*Capillospirura ovotrichuria*
Sphaerospora colomani	*Cystoopsis acipenseris*
Hexamita truttae	*Leptorhynchoides plagicephalus*
Costia necatrix	*Pomphorhynchus laevis*
Apiosoma spp.	*Piscicola geometra*
Chilodonella cyprini	*Unionidae* gen. sp.
Coleps spp.	*Argulus foliaceus*
Ichthyophthirius multifiliis	*Chaetogaster limneai*
Polipodium hydriforme	

Of protozoan infections which should perhaps be considered the most important from the viewpoint of intensive fish culture, I have thoroughly studied the development, morphology and pathology of the myxosporean parasites *Sphaerospora colomani* and *Chloromyxum inexpectatum*, as before this study no data except the species descriptions had been available on these parasites.

Development of *Sphaerospora colomani*

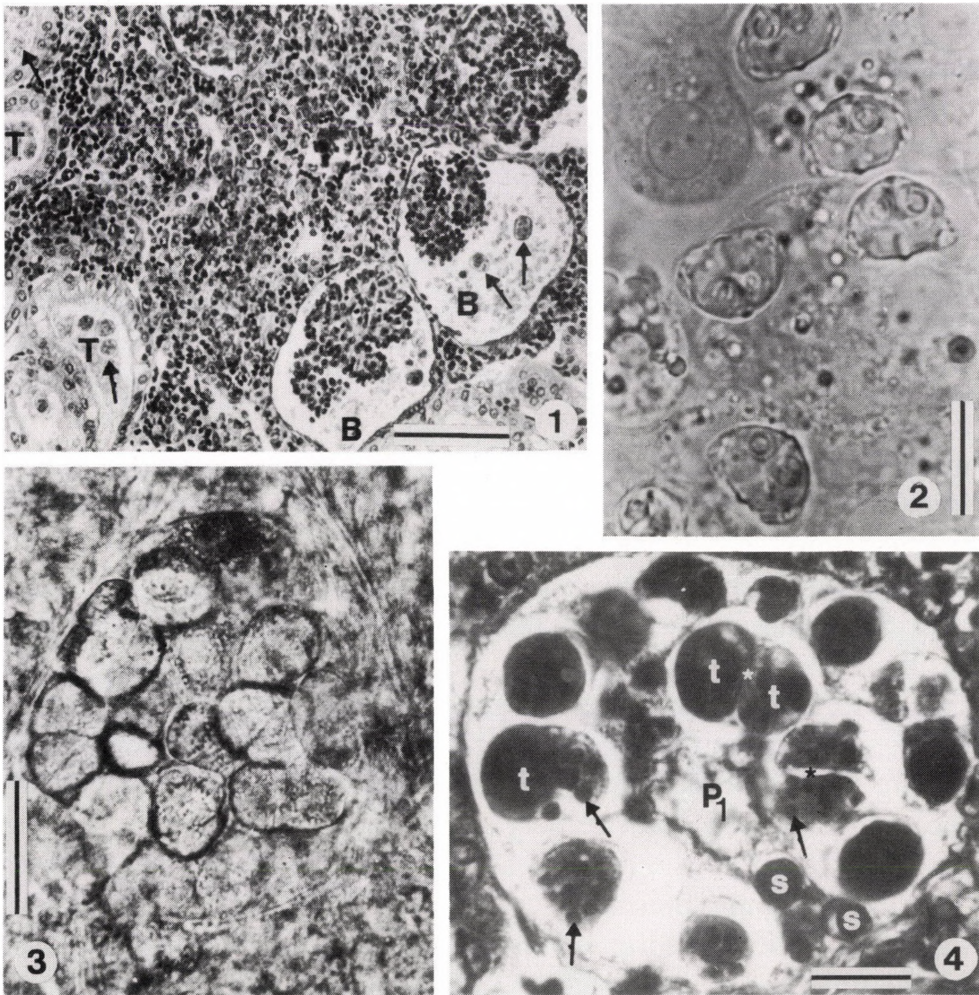
This myxosporean sterlet parasite forms spores in bisporoblastic pseudoplasmodia located in the Bowman's space and in the upper urinary passages, in the lumen of tubules (Fig. 1). By the characteristic shape and size of the spores (Fig. 2), the species can be easily distinguished from other sphaerospores described in the literature so far. Spore formation takes place in late spring and in the first summer month, when large numbers of pseudoplasmodia loosely attached to the tubular epithelial cells can be found in the renal tubules both in fresh preparations and in sections. These pseudoplasmodia cause marked dilatation of the tubular lumen (Fig. 1).

It is known from the literature (Baska and Molnár, 1988) that sphaerosporosis of cyprinids is accompanied by the presence of a so-called "C blood protozoon" showing active movement in the blood. This "C blood protozoon" was first described by Csaba (1976), and identified as a developmental stage of sphaerospores by Molnár (1988). This blood stage precedes spore formation but occasionally persists in the blood in large numbers and can be found in the capillaries throughout the body, first of all in the swimbladder, eye fundus and gills also after sporogenesis has passed off. I assumed that a presporogonic stage ensuring excessive parasite multiplication within the fish organism must exist also in the case of *Sphaerospora* infection of the sterlet. Therefore, I regularly looked for a similar blood stage in blood smears and by the enrichment method described by Sövényi and Molnár (1990). Parallel to that, I examined fresh impression smears made of the different organs for the presence of blood stages in the capillaries. I could not find blood stages similar to those described for cyprinids either in the blood smears examined in fresh state and after staining, or by studying the content of capillaries. At the same time, in fresh squash preparations of the kidney examined in the early autumn period I regularly found an until then unknown formation of complex structure (Fig. 3). My aim was to subject that formation to histological and electron microscopic studies to determine if it may be the pre-sporogenic form of some myxosporean parasite.

As seen in fresh preparations, by the end of October these structures had reached 50–70 μm in size, were spherical in shape, and were demarcated from the surrounding tissues by a thin membrane. Most of the structures contained 30–40 transparent globules 8–12 μm in size and divided into two halves by a septum running in the middle (Fig. 3). With the passing of winter, these structures disappeared from the kidneys and, at the same time, the sterlet population living in natural waters developed intensive sphaerosporosis.

The youngest developmental stages appeared in the renal parenchyma and were located in the cytoplasm of the endothelial cells. The parasite, in the present case the primary cell, began to grow and substantially dilated the attacked host cell. Simultaneously with that, secondary cells appeared and multiplied in the cytoplasm of the primary cell. By the end of October, also the secondary cells became differentiated and two tertiary cells appeared in their cytoplasm. When examined by oil immersion, the intensely staining cytoplasm of the tertiary cells was found to contain numerous quaternary cells and free nuclei (Fig. 4). Besides their typical intertubular location, the vegetative stages of *Sphaerospora colomani* occasionally occurred also in the haematopoietic tissues.

In order to get a closer insight into the details of development and location, I have performed electron microscopic studies. At low magnification, the cytoplasm of the primary cell was found to contain secondary cells showing various stages of development; in the secondary cells, the cross-sections of tertiary cells were seen (Fig. 5). The nucleus of the primary cell was usually located laterally and enlarged like a vesicle, while its cytoplasm was electron light and contained an endoplasmic reticulum network, free ribosomes and numerous tubular mitochondria as well as secondary cells demarcated from the cytoplasm of the primary cell by a unit membrane having numerous surface-increasing projections. The younger secondary cells had distinct and heterochromatic nuclei. The cytoplasm of the more developed cells contained tubular mitochondria, electron-dense nutrient vesicles, free ribosomes and a usually peripherally located heterochromatic nucleus, with a pronounced nucleolus. By internal



Figs 1–4. Sphaerospora colomani Baska, 1990. *Fig. 1.* Pseudoplasmodia (arrows) developing in Bowman's space (B) and in the tubular lumen (T). Bowman's space and tubular lumen are markedly dilated. Haematoxylin and eosin (H-E). Bar = 100 μm. *Fig. 2.* Mature spores of *S. colomani*. Bar = 10 μm. *Fig. 3.* Developing vegetative stages in a fresh squash preparation of the kidney containing numerous dividing units inside a membrane envelope. Bar = 20 μm. *Fig. 4.* Histological section of the previous young stage. There are secondary cells (s) embedded in the primary plasm (P_1). Beside the secondary cells there are paired stages (tertiary cells = t) inside vacuoles, each containing several nuclei and tiny cells (arrows) that stain intensively with haematoxylin. (*) septum. H-E. Bar = 20 μm.

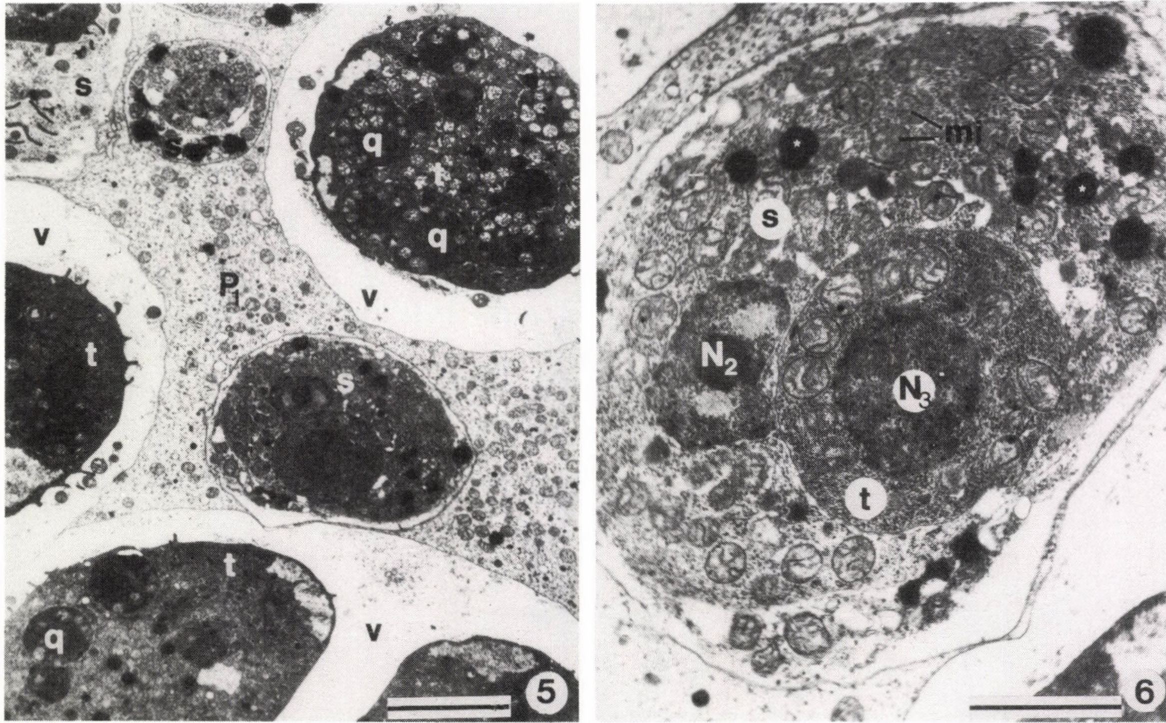
cleavage, in the cytoplasm of the secondary cell a tertiary cell develops (Fig. 6). This tertiary cell then begins to grow and differentiate, divides into two, and gives rise to quaternary cells which complete the cycle of vegetative reproduction. Parallel to these processes, the cytoplasm of the secondary cell becomes rarefied and disintegrates, and the secondary cell itself changes into a vacuole which contains only cellular debris and two intensively developing tertiary cells (Fig. 7).

The cell membrane of the tertiary cells also bears finger-like projections. Their electron-dense cytoplasm rich in structural elements contains free nuclei and occasionally more than ten quaternary cells bounded by membrane and having a nucleus of their own. As they are markedly electron dense, their fine structure is difficult to study. At the same time, their limiting membrane, the relatively large nucleus with the nucleolus, and the few tubular mitochondria located in the narrow semi-circle of cytoplasm are well distinguishable (Fig. 7).

Summing up the development of *Sphaerospora colomani* in the tissues, it can be established that, in the absence of blood stages, this species forms vegetative presporogonic stages in primary cells located in the endothelium of intertubular capillaries of the kidney. Completing their reproduction and upon disruption of the primary and secondary cells and of the tertiary cells harbouring them, these spores enter the blood stream and reach the typical site of spore formation (the cavity of the Bowman's capsule and the lumen of the renal tubules) where they form spores in bisporoblastic pseudoplasmodia. As the final stage of division seen in the renal tissue is the quaternary cell which does not differentiate further, it is probably this stage which forms the bisporoblastic pseudoplasmodia upon reaching the lumen of renal tubules.

Development of *Chloromyxum inexpectatum*

Almost all sterlets dissected during the parasitological faunistic survey that has been going on for several years harboured *Chloromyxum inexpectatum* infection. The spores of the parasite can be



Figs 5–6. Sphaerospora colomani Baska, 1990. *Fig. 5.* Electron micrograph of a vegetative stage of *S. colomani*. Membrane-lined secondary cells (s) embedded in the primary plasm (P_1). In the more differentiated secondary cells, two tertiary cells (t) appear, which have strongly electron-dense cytoplasm comprising many mitochondria and several quaternary cells (q). The tertiary cells are situated in vacuoles (v) inside the disintegrated secondary cells. Bar = 5 μm. *Fig. 6.* Inside the secondary cell (s) close to the nucleus (N_2) a young tertiary cell (t) can be seen with tubular mitochondria (mi) and a relatively big, euchromatic nucleus (N_3). The plasm of the secondary cell contains tubular mitochondria, free ribosomes and electron-dense nutrient vesicles (*). Bar = 2 μm.

found in large numbers in the lower urinary passages where the polysporoblastic pseudoplasmodia adhere to the urothelium (Fig. 8) and form spores throughout the year, although at a rate decreasing with time. Pseudoplasmodia 20–250 μm in size, containing refractile granules measuring 2–3 μm, are almost always seen in fresh squash preparations and scrapings obtained from the mucous membrane of the urinary passages. Depending on their size, these pseudoplasmodia contain one or more developing spore(s). The released mature spores are bullet shaped and comprise four nearly spherical polar capsules located in the apical part of the spore (Fig. 9). The wall of the spore consists of two valves united by a concentrically running suture (Baska, 1990).

In my paper presenting the description of the species (Baska, 1990), I mentioned the presence of a cellular structure of unknown function and origin in the capillary network of the glomerulus. I observed this phenomenon more and more frequently. It has turned out that the first "infected" glomeruli, the presence of which can in most cases be demonstrated by histological examination only, appear at the beginning of autumn (Fig. 10). With time, these formations begin to grow vigorously, markedly distorting the glomerular structure: the capillary network is pushed to the periphery and cells of unknown origin proliferate in the centre. At that stage, the forms are already visible also in fresh squash preparations examined by light microscopy. At the end of January, the glomerulus is already entirely unrecognizable and is replaced by an enormous cell mass 150–200 μm in diameter, consisting of more or less equally refractile cells. By light microscopy, no further changes are demonstrable until the end of February when these structures abruptly disappear from the renal tissue. Some time later, the young pseudoplasmodia of *Chloromyxum inexpectatum* attached to the epithelial cells appear in the lower urinary passages.

Not knowing the origin of the phenomenon, by its light-microscopic structure I named the formation a large structure of syncytial organization. When examined after staining with haematoxylin and eosin, the syncytial structure was surrounded by a narrow,

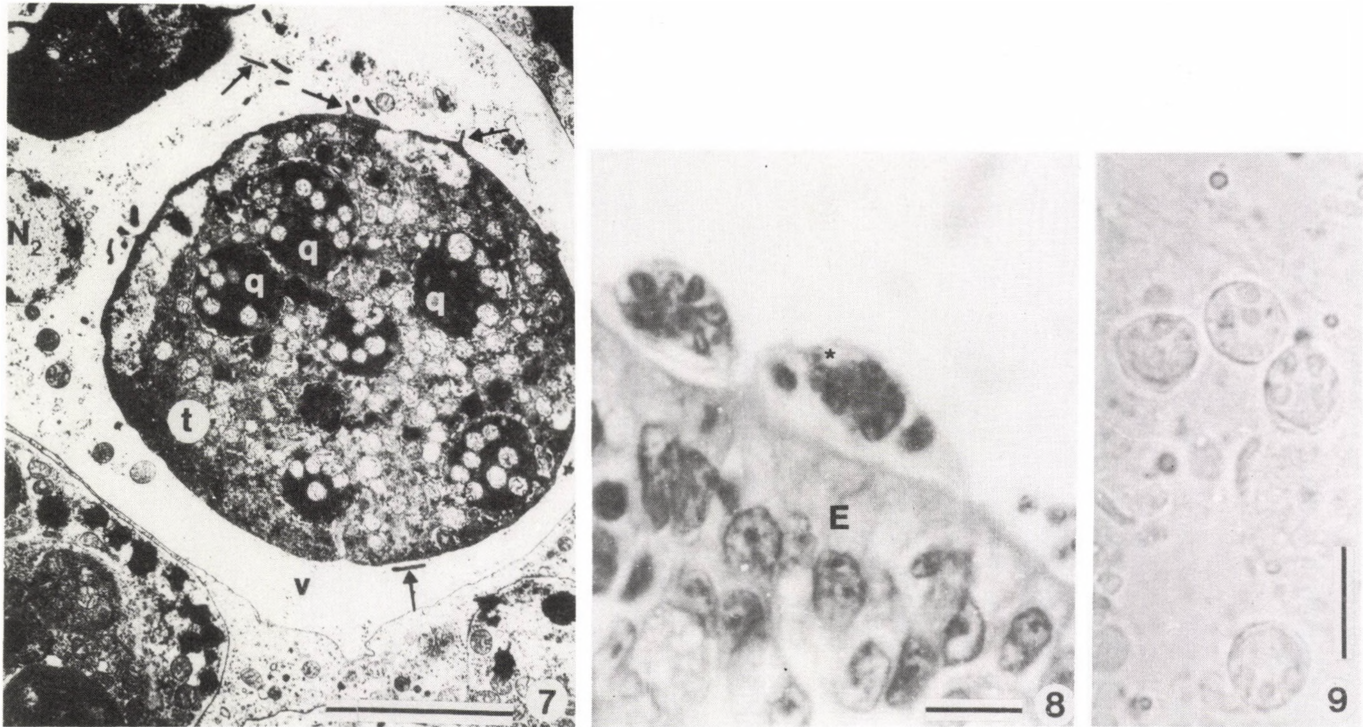
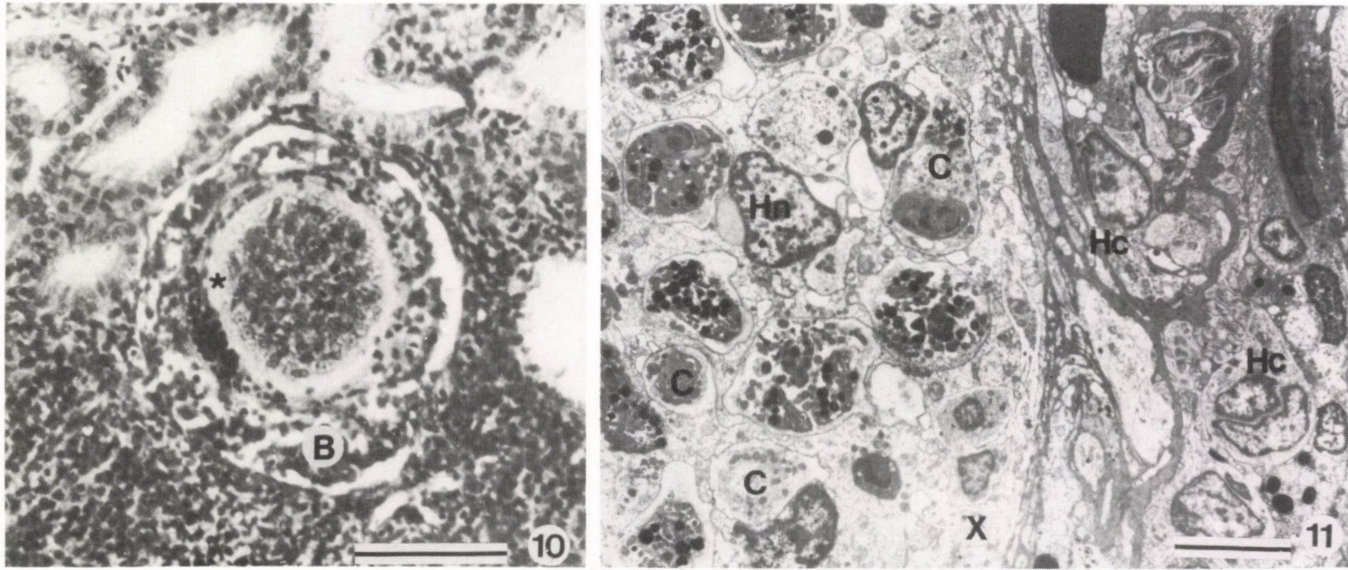


Fig. 7. Sphaerospora colomani Baska, 1990. In a membrane-lined vacuole (v) a puffed nucleus (N_2) and some tubular mitochondria can be seen, which are the remnant of the secondary cell plasm. The full cross-section of the tertiary cell (t) shows the structure of the cell and the quaternary (q) cells. The tertiary cells have finger-like processes on their surface (arrows). The quaternary cells are strongly electron-dense and they contain tubular mitochondria and a heterochromatic nucleus. Bar = 5 μm *Figs 8–9. Chloromyxum inexpectatum* Baska, 1990. *Fig. 8.* Spore-forming pseudoplasmodia (*) adhering to the epithelial cells (E) in a thin layer. Bar = 10 μm. *Fig. 9.* Immature spores of *C. inexpectatum*. Bar = 10 μm.

eosinophilic, homogeneous band in which large numbers of nuclei were crowded together, with indistinct cell borders or without any cell borders whatsoever (Fig. 10).

Myxosporean infections of the Bowman's capsule have been described by Dykova et al. (1987), who reported the histological and electron microscopic findings obtained on the sporogenesis of *Myxidium rhodei*, Léger 1905, and by Lom et al. (1989), who published valuable data on the ultrastructure of a xenoma formed in the kidneys by the pike parasite *Myxidium lieberkuehni*, Bütschli 1882. Infection of the Bowman's capsule by different developmental stages of further myxosporeans (*Myxidium giardi*, *Sphaerospora epinepheli*, *Ceratomyxa hungarica* etc.) has been reported in numerous other cases (Copland, 1983, Supamattaya et al., 1991, Molnár, 1992). In order to determine whether the structure found by Baska (1990) was an anatomical formation or represented an early developmental stage of some parasitic protozoan (e.g. myxosporean), I performed electron microscopic studies.

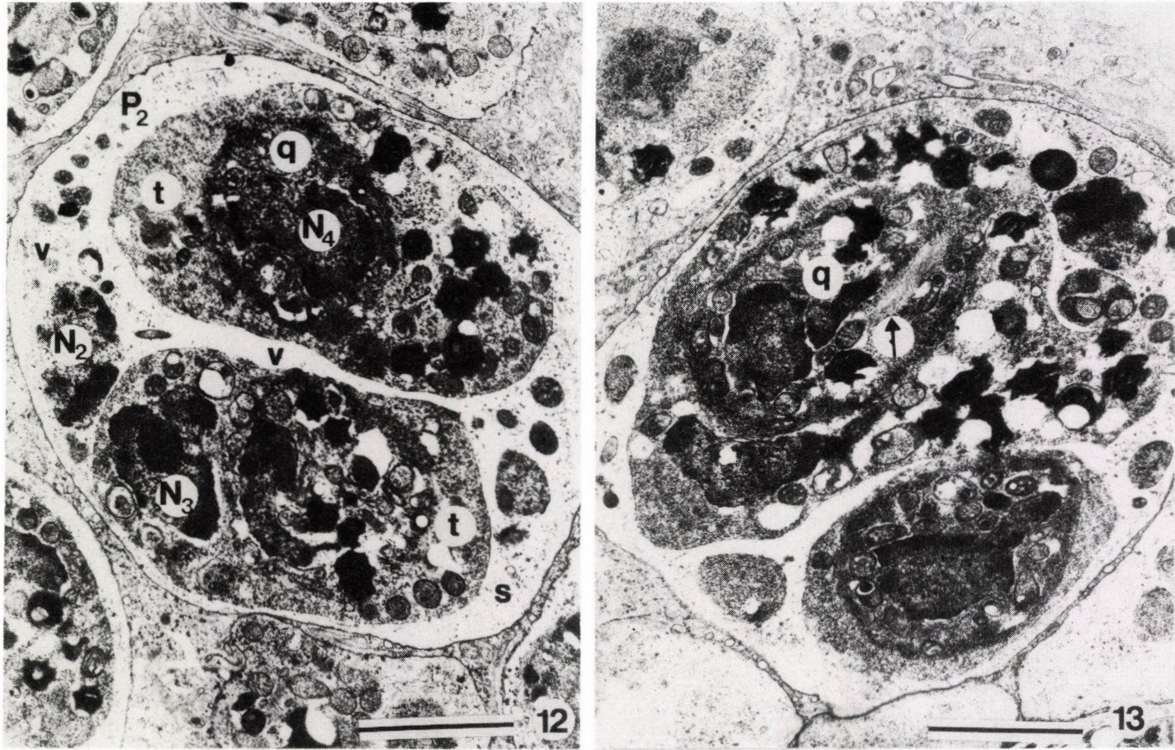
The parasitic origin of the structure was soon established by examining ultrathin sections prepared from the area containing the infected glomeruli, already at low magnification (Fig. 11). In the centre of the glomerular capillary network, a xenoma demarcated by two rows of host cells and a narrow electron-light zone was seen, formed by the combination of parasitic cells showing typical internal cleavage and the large, euchromatic nuclei of host cell origin. From the outside, the xenoma was bordered by a layer consisting of two rows of host cells, which probably develops from the podocytes of the Bowman's capsule. Within the cell rows, there was a zone of reticular structure which histologically appeared to be a homogeneous, eosinophilic layer. Electron microscopic examination revealed that it was composed of tubules, vesicles and laminar membrane structures. The actual xenoma-cytoplasm, which contained different developmental stages of the parasite, joint nuclei of the xenoma, nutrient vesicles, tubular mitochondria, an endoplasmic reticular system and free ribosomes, was situated within that layer.



Figs 10–11. Chloromyxum inexpectatum Baska, 1990. *Fig. 10.* The young stages of *C. inexpectatum* develop inside a xenoma among the capillary loops of the Bowman's capsule (B). The xenoma is surrounded by an eosinophilic, homogenous zone (*). Bar = 100 μm. *Fig. 11.* A particular xenoma (X) is formed in the Bowman's capsule from the vegetative stages of the parasite (C) and from big, euchromatic nuclei (Hn) of host cells. The xenoma is delineated and nourished by a capsule of host cell (Hc) layers. Bar = 5 μm.

The developmental stages of the parasite are represented by the secondary cells including two tertiary cells each. Within the cytoplasm of each tertiary cell, a quaternary cell is formed by internal cleavage. The secondary cells multiply by simple division. Subsequently, a tertiary cell is formed in their cytoplasm by internal cleavage, which tertiary cell undergoes further bipartition (Fig. 12). The cytoplasm of the secondary cell contains mitochondria, free ribosomes and markedly electron-dense reserve nutrient droplets of irregular shape. The nucleus of the secondary cell is large, vesicle-like, dilated and euchromatic. The nucleolus is distinct. The cytoplasm of the young tertiary cell is abundant in mitochondria and surrounds the heterochromatic nucleus ringwise. The secondary cell disintegrates parallel to the complete development of the tertiary cells: its nucleus becomes pycnotic and is pushed to the periphery, its cytoplasm becomes rarefied and the cell organelles undergo disruption. Inside the secondary cell membrane two fully formed tertiary cells can be seen (Fig. 12) with an electron-dense cytoplasm filled with cell organelles and reserve nutrients, and with a chromatin-rich, peripherally located nucleus. In the centre of each tertiary cell a quaternary cell is located: this quaternary cell is composed of a relatively large, amorphous nucleus and an electron-dense cytoplasm containing numerous mitochondria. The quaternary cells frequently contained longitudinally running tubular structures (Fig. 13) which often emitted long projections toward the surface and, thus, assumed a flagellum-like shape. A possible role of this structure in the active movement of presporogonic stages cannot be ruled out.

Summing up the knowledge obtained on the development of *Chloromyxum inexpectatum* in the tissues, it can be stated that this myxosporean parasite forms a xenoma in the capillary network of the Bowman's capsule. The xenoma reaches full development during the winter. Its cytoplasm is controlled by numerous large nuclei of host cell origin, among which parasitic cells of different developmental status are found. The basic unit is the secondary cell which forms a tertiary cell within its cytoplasm by internal cleavage. The tertiary cell undergoes bipartition within the secondary cell which gradually loses



Figs 12–13. Chloromyxum inexpectatum Baska, 1990. *Fig. 12.* The characteristic mode of multiplication of the secondary cell (s) is the internal cleavage. The disintegrated secondary cell forms a vacuole (v) around the two tertiary cells (t). The rudimentary nucleus (N_2) and the remnant of the secondary cell plasm (P_2) surround the tertiary cells, which have peripherally located nuclei (N_3) and cytoplasms densely filled with nutrients (*). In the centre of the tertiary cell a quaternary cell (q) is visible with a strongly electron-dense nucleus (N_4). Bar = 2 μm. *Fig. 13.* A complex, longitudinally arranged tubular structure (arrow) could often be observed in the quaternary cells (q). Bar = 2 μm

its structural integrity and function parallel to the intensive development of tertiary cells. Each tertiary cell forms a quaternary cell by internal cleavage. The electron microscopic findings show that during the tissue phase the quaternary cells are the most differentiated forms; these quaternary cells probably induce the formation of polysporoblastic pseudoplasmodia upon reaching the urinary passages and adhering to the epithelial cells. The mode of their excretion is simple: upon reaching maturity, the xenoma bursts and its content is released into the Bowman's capsule and, from there, into the lumen of tubules.

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MYCOPLASMAL ARTHRITIS AND MENINGITIS IN CALVES

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A disease entity slightly different from, and appearing at an earlier age than, the syndrome known as mycoplasmal pneumo-arthritis was diagnosed in sucking calves. The disease, which had probably originated from intrauterine infection and manifested itself as polyarthritis occasionally accompanied by meningitis, caused huge economic losses due to a substantial reduction of the calf crop on the farm involved. Severe clinical signs were often observed already in the first days after birth. Besides an elevated body temperature reaching 40–41 °C, the affected calves showed striking deformities which made most of them unable to stand up or suck; as a result, they became emaciated and died at an age of 1–3 weeks. The gross pathological picture was dominated by serofibrinous arthritis involving several joints. In the articular cavities a large volume of straw-coloured, transparent synovial fluid accumulated which contained thick, confluent, white fibrin films. In some cases a similar exudate was observed in the thoracic cavity, pericardium and cerebral ventricles, too. Histopathological examination revealed serofibrinous inflammation of the synovial membrane of the joints, the pericardium, the pleura, the leptomeninges and the ependyma lining the cerebral ventricles. Our diagnosis was confirmed by the electron-microscopic detection of mycoplasmas from the pathologically altered organs and the isolation of a pathogen identified as *Mycoplasma bovis.*

Key words: Mycoplasma, calf, meningitis, arthritis, pathology

Up to the present, 15 *Mycoplasma* and *Acholeplasma* species have been reported from cattle. Of them, *Mycoplasma mycoides* (the small colony type) is associated with a well-defined disease called bovine pleuropneumonia (Cottew, 1979). *M. bovis* can cause pneumoarthritis in calves (Allan et al., 1978; Gourlay et al., 1976; Langford, 1976; Romváry et al., 1977; Pfützner et al., 1980; Ryan, 1982), mastitis (Bennett and Jasper, 1977; Boughton and Wilson, 1978; Horváth et al.,

1983; Pfützner et al., 1983; Pfützner and Schimmel, 1985) and abortion in cows (Stalheim and Proctor, 1976), impaired sperm motility (Jurmanova and Sterbova, 1977) and, rarely, keratoconjunctivitis (Jack et al., 1977). *M. bovigenitalium, M. californicum* and *M. canadense* have been implicated in the aetiology of mastitis in cows and of urogenital disorders in cows and bulls (Counter, 1978; Ernø, 1975; Gourlay et al., 1978; Jasper et al., 1979; Mackie and Logan, 1982). *Ureaplasma diversum* can cause pneumonia in calves and urogenital disorders (Doig et al., 1980; Muenster et al., 1979; Waelchli-Suter et al., 1982). *M. dispar* has also been associated with calf pneumonia (Friis, 1980; Muenster et al., 1979; Tinant et al., 1979). Other species inconsistently participate in different disease entities: *M. bovirhinis* and *Acholeplasma modicum* in respiratory diseases (Jurmanova et al., 1975; Stipkovits, 1973) while *M. alcalescens* in arthritis (Bennett and Jasper, 1978). Arthritis may be associated, besides some of the species mentioned above, also with unidentified mycoplasma strains.

Most authors have implicated *M. bovis* infection in the aetiology of severe fibrinous-purulent arthritis of 3-week to 4-month-old calves. However, few reports are available in the literature on mycoplasma-induced arthritis and pulmonary processes associated with diseases of the central nervous system (Bennett and Jasper, 1978). Also, there is a scarcity of data on mycoplasmal arthritis occurring in day-old calves, originating from intrauterine infection.

This paper reports epizootiological, pathological and diagnostic observations concerning serofibrinous arthritis accompanied by pneumonia and meningitis of mycoplasmal origin in 3-day to 3-week-old calves.

Materials and methods

Test material. Calves that had died of natural infection in two large cattle herds during a 4-month period of the past year were examined. In one of the herds (A), on-the-spot investigations were conducted simultaneously with the laboratory diagnostic examinations. A

total of 8 calves of the two herds were subjected to pathological examination.

Histological and electron-microscopic examination. Samples were taken from the liver, lungs, brain, pericardium, synovial fluid and synovial membrane, fixed in 5% buffered formalin and stained with haematoxylin and eosin for general information and sometimes also according to Giemsa and Stamp. For electron-microscopic examination, samples were taken from the synovial fluid and the fibrinous exudate present in the articular cavity. The synovial fluid was processed by negative staining with phosphotungstic acid and the fibrinous exudate was fixed in 2.5% glutaraldehyde followed by 1% osmium tetroxide. After embedding in Durcupan, ultrathin sections were cut with an ultramicrotome, counterstained with uranyl acetate and lead citrate, and examined in a Philips 201 CS electron microscope.

Microbiological examination. For microbiological examination, samples were taken from the spleen, lungs, brain, synovial fluid, small intestinal content and, occasionally, pericardial exudate and umbilical stump. The samples were streaked on 5% sheep blood agar, common agar and Drigalski's as well as Minca's solid media. After incubation at 37 °C for 24–72 h, the cultures were evaluated daily and bacteria were identified by the methods described by Cowan (1974). For isolation of mycoplasmas, samples from the lungs and the inflammatory exudate that accumulated in the articular cavities, pericardium and brain were inoculated into liquid medium B (Ernø and Stipkovits, 1973). Cultivation and biochemical as well as serological identification (growth and metabolic inhibition, epifluorescence test) were done as described elsewhere (Stipkovits, 1973). The jejunal and ileal contents were checked for the presence of rota- and coronaviruses by immunofluorescence. In less than 2-week-old calves, scrapings taken from the wall of the small intestine were dried, fixed in methanol, stained by Castaneda's method and examined by microscopy for the presence of cryptosporidia. For the demonstration of chlamydiae, smears were made from the lungs, tracheal mucosa, pericardium and synovial fluid, fixed over flame, stained according to Stamp, and evaluated by light microscopy.

Results

Case history. Epizootiological observations. Herd A was a dairy herd of 800 animals including 107 pregnant heifers. Newborn calves were kept in individual pens up to 5 days of age, then in the prophylactorium in groups of 6. Subsequently, the calves were transferred to the rearing house where they were kept in groups of 15 for 2–3 weeks. Calf rearing lasted for 7–8 weeks. The cows were inseminated with the semen of three bulls.

In herd A, the first disease cases occurred among calves in the calving house on the 3rd day of life. Clinical signs included depression, elevated body temperature, swelling of one or more extremital joints, and mild coughing. Less frequently, nervous signs (opisthotonus and swimming-crawling limb movements) also occurred. Neither diarrhoea nor umbilical infection was observed. In many cases, well-developed, healthy, viable calves which had suckled their dams normally for some days, collapsed abruptly without any previous clinical signs, could not stand up, and died at 3–5 days of age in spite of treatment. Calves that survived the critical first week showed some transient improvement for 2–3 days but, due to deformation of their joints, could neither stand up nor suckle their dam, became emaciated and died of chronic polyarthritis and, sometimes, pneumonia at 2 or 3 weeks of age. Death was preceded by fever (40–41 °C), coughing and scours of short duration (excretion of watery, blood-stained faeces). The therapy applied (Linco-spectin, Tylan, Terramycin LA) failed to reduce the severity of clinical signs and the mortality rate. At the beginning, 20 out of 80 newborn calves died (25% mortality rate). One month later the mortality rate increased to 50%. During the 2-month period of observation, a total of 178 calves were born and the stillbirth rate was 3.9%. All affected calves had been derived from dams inseminated with the semen of two of the three bulls used. The offspring of the third bull remained healthy throughout. For two years, the cows had shown sporadic but periodically recurring cases of mastitis, with the secretion of blood-stained milk by one or more udder quarters. These cows were regularly removed from the herd and kept isolated.

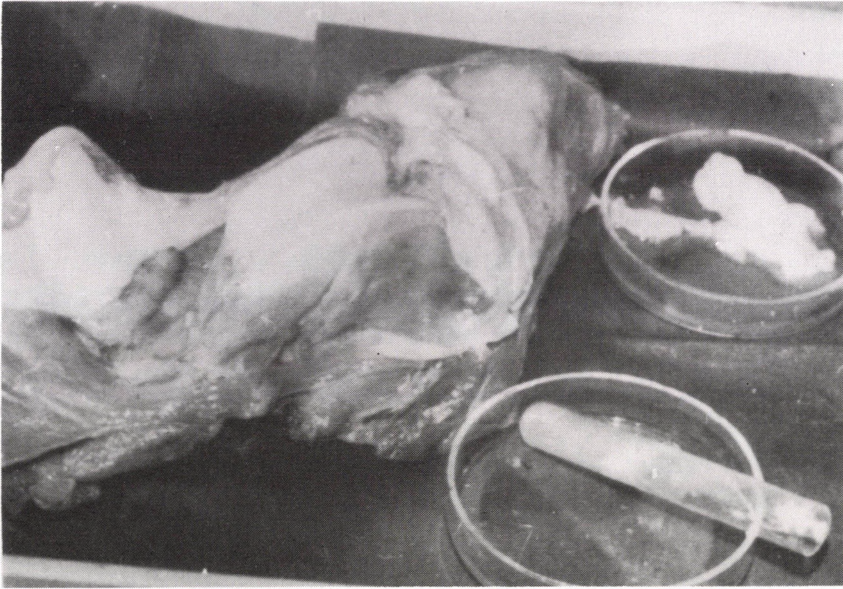

Fig. 1. Accumulation of serum and fibrin in the articular cavity

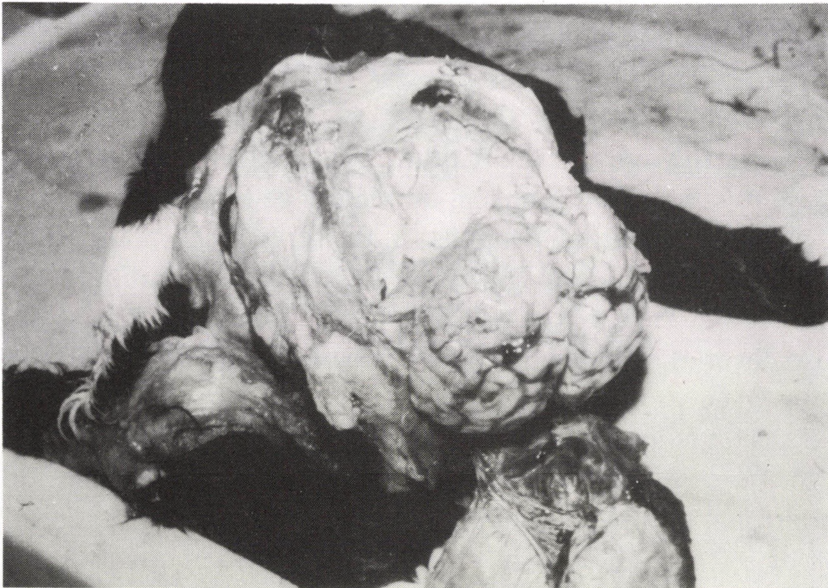

Fig. 2. Fibrinous meningitis

From herd B, calves that had died of diarrhoea at day-old as a result of a suspected rearing disease were submitted for examination. Gross pathological and complementary routine diagnostic examinations revealed arthropathies.

No details are available on herd B.

Gross and histopathological examination. At necropsy, all calves were found to have severe serofibrinous arthritis of all extremital joints and, in many cases, also of the hip and sacral joints. The knee, tarsal and carpal joints were swollen, deformed and undulating to the touch. They contained large amounts of straw-coloured, transparent synovial fluid with thick, confluent, white fibrin films floating in it (Fig. 1). Sometimes a similar exudate was found in the thoracic cavity, pericardium and cerebral ventricles (Fig. 2). Ulceration of the synovial membrane was also observed in a few calves. In 3 to 18 days old calves the gross pathological picture was dominated by the joint lesions described above. In addition, swelling and hyperaemia of the liver, focal bronchopneumonia as well as acute abomasitis and enteritis were found. In a calf that had died at the age of 3 days, catarrhal-purulent pneumonia accompanied by extensive fibrinous pleuritis and pericarditis was found. Fibrinous meningitis was seen in an animal that had died at 8 days of age.

Histopathological examination revealed fibrin filaments and infiltration consisting of different inflammatory cells (lymphocytes, histiocytes, plasma cells and neutrophils) in the serous membranes including the synovial membrane (Figs 3 and 4), the pericardium and the pulmonary pleura. Here and there, the surface of the serous membranes was also covered by a serofibrinous exudate. In one calf, similar lesions were observed in the leptomeninx and the ependyma lining the cerebral ventricles (Figs 5 and 6).

In the case of mild, macroscopically hardly observable lung lesions, histopathology revealed an incipient catarrhal infiltration of the alveoli of a few lobules, with pronounced hyperaemia and accumulation of an exudate consisting of detached epithelial cell debris. Some alveolar and bronchial macrophages contained particles corresponding to mycoplasmas in size (250–300 nm) and staining properties. In two

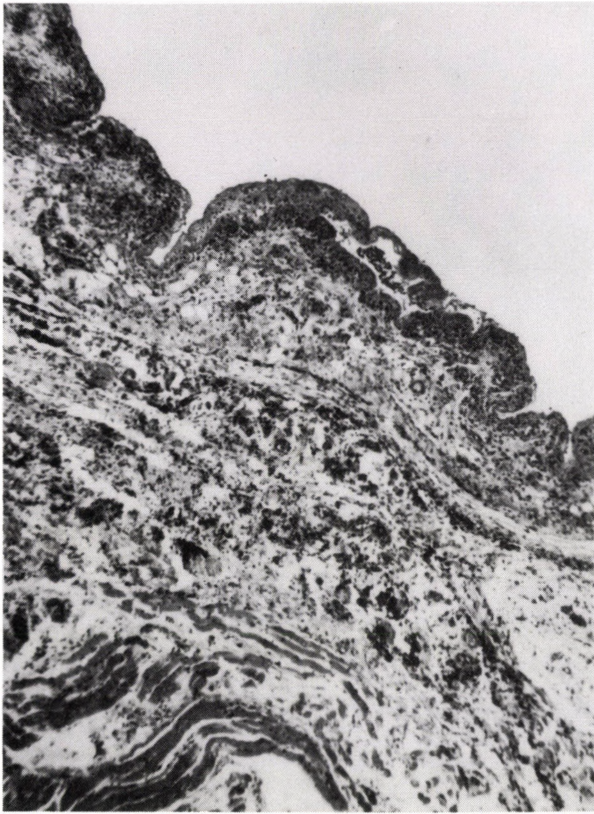

Fig. 3. Thickening and inflammatory infiltration of the synovial membrane in a calf showing swelling of the carpal joints. Haematoxylin and eosin (H.–E.), x 40

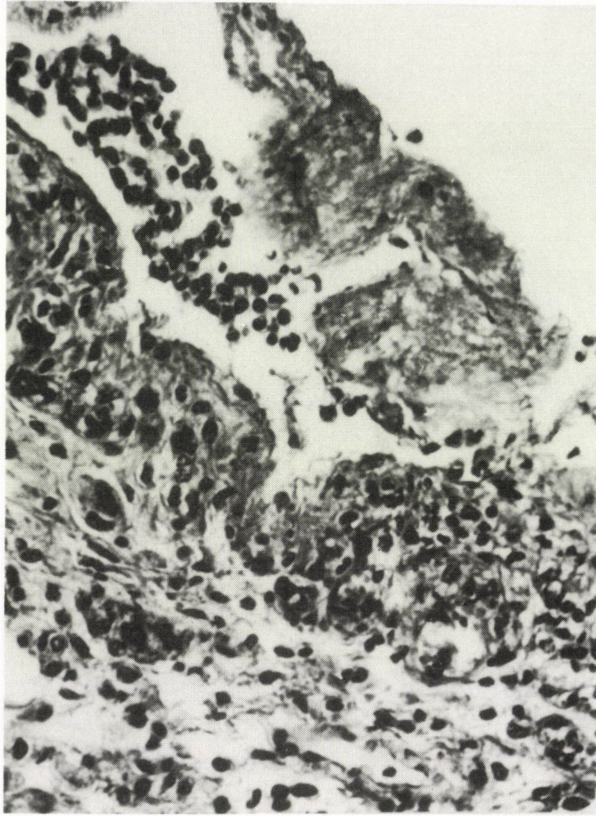

Fig. 4. Detail of Fig. 3. The synovial membrane is infiltrated by inflammatory cells and serofibrinous exudate. H.–E., x 160

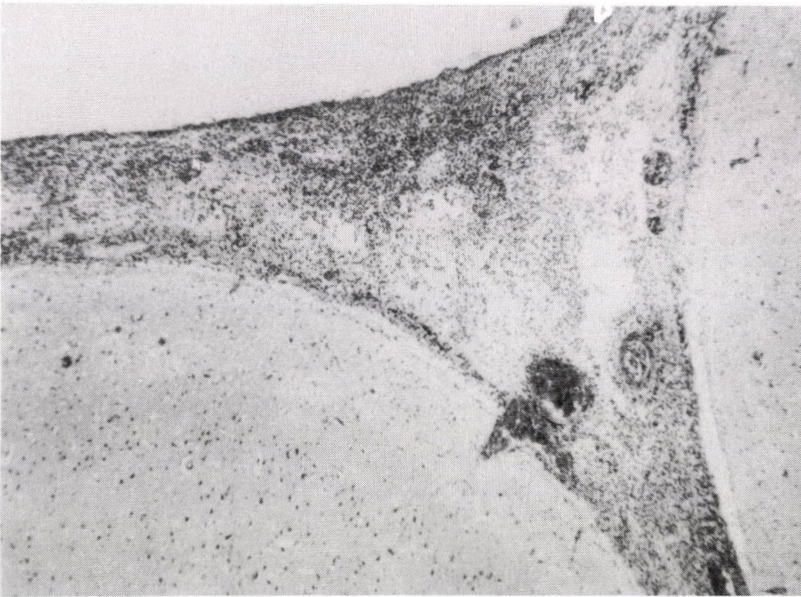

Fig. 5. Thickening and inflammatory infiltration of the leptomeninx in a calf showing nervous signs. H.–E., x 40

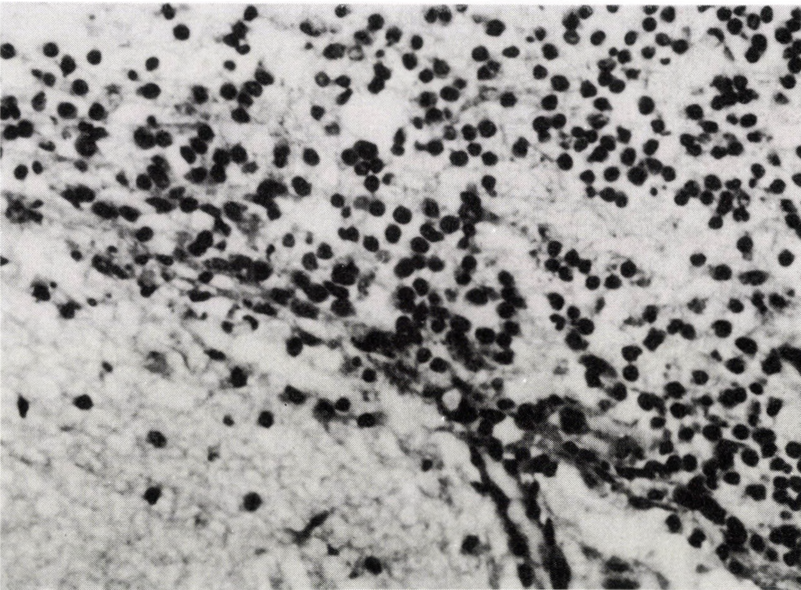

Fig. 6. Detail of Fig. 5. Thickened leptomeninx infiltrated by lymphocytes, histiocytes, granulocytes and serofibrinous exudate. H.–E., x 160

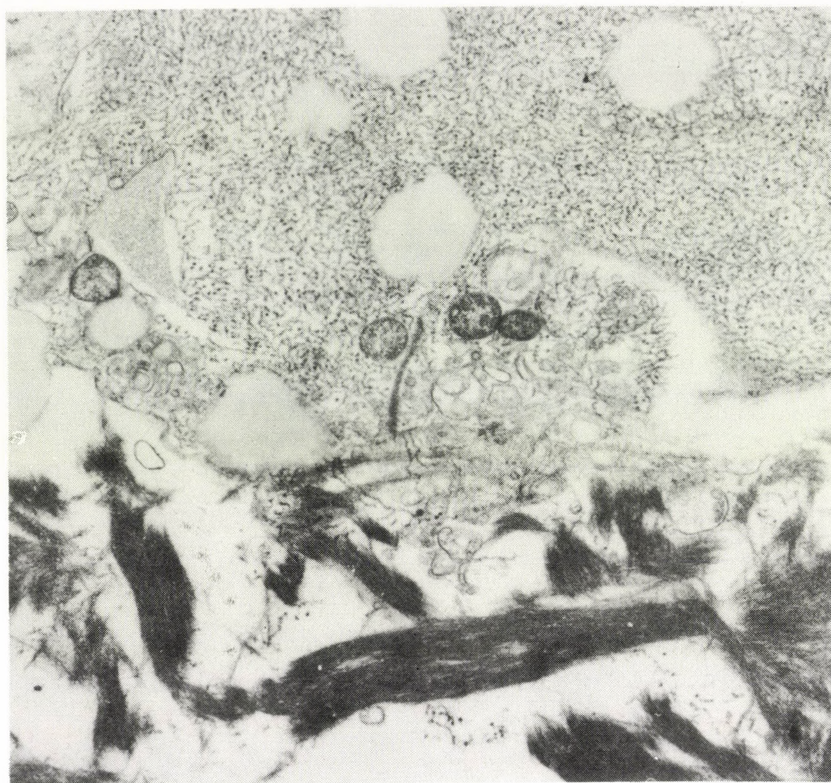

Fig. 7. Mycoplasmas among the fibrin filaments in the fibrinous exudate that accumulated in the articular cavity. Electron micrograph, x 9,600

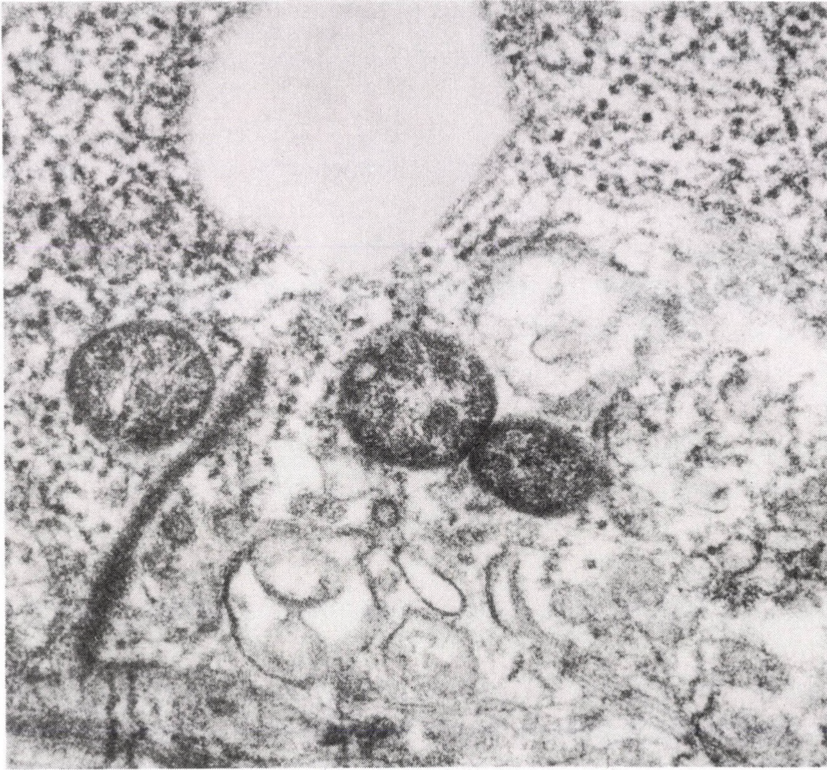

Fig. 8. Detail of Fig. 7. Mycoplasma particles 250–300 nm in diameter, bounded by double membrane and lacking a cell wall. Electron micrograph, x 30,000

cases of pneumonia macroscopically judged to be severe, histopathological examination revealed catarrhal-purulent bronchopneumonia.

In the liver, activation and focal proliferation of cells belonging to the MPS, sometimes associated with centrolobular parenchymal degeneration, incipient necrosis of hepatocytes and haemorrhages as well as an incipient serous hepatitis accompanied by elevated leucocyte counts were demonstrated.

Electron microscopy of the synovial fluid and the fibrinous exudate that accumulated in the articular cavity showed the presence of inflammatory cells (lymphocytes, histiocytes and granulocytes) originating from the blood and particles 250 to 300 nm in diameter, bounded by a double membrane and lacking a cell wall, which corresponded to mycoplasmas in ultrastructure (Figs 7 and 8), among the fibrin filaments. Neither viral particles nor bacterial forms were demonstrated.

Microbiological examination. Mycoplasmas were isolated from the synovial fluid, the lungs and the fibrinous exudate that accumulated in the cerebral ventricles. On the basis of their biochemical characteristics and the results of the growth inhibition, metabolic inhibition and epifluorescence tests, the isolates were identified as *M. bovis.*

Attempts to isolate bacteria from the spleen and brain of calves by the usual bacteriological procedures consistently failed. Lung areas showing signs of complicated catarrhal-purulent pneumonia yielded *Pasteurella multocida.* The aerobic intestinal microflora consisted of mucoid-type K99^{-} *Escherichia coli.*

Virological examination of the intestinal content for rota- and coronaviruses and its microscopic examination for cryptosporidia gave negative results, similarly to that of the synovial fluid for chlamydia.

Discussion

In suckling calves submitted from two different large herds for diagnostic examination, we demonstrated a disease entity which is

somewhat different from the known mycoplasmal pneumoarthritis of calves and occurs at a younger age.

Earlier experience (Gourlay et al., 1976; Romváry et al., 1977; Bennett and Jasper, 1978; Pfützner et al., 1980; Ryan, 1982) indicates that mycoplasma-induced arthritis of calves mainly occurs between 3 weeks and 4 months of age and is preceded by a more or less severe pneumonia. In contrast, in the cases presented here the lesions caused by mycoplasma infection were diagnosed in 3- to 21-day-old calves.

According to the case history data, the clinical signs of severe arthritis (in most cases polyarthritis) and concomitant pneumonia, accompanied by nervous signs, had already been observed in the calving house, on the third day of life.

Characteristic lesions were found in the calves that had become ill and died. The pathological picture was dominated by severe serofibrinous arthritis involving several joints. Mycoplasma infection could be diagnosed with high reliability already by gross and histopathological examination. The diagnosis was confirmed by electron-microscopic demonstration of mycoplasmas in the pathologically changed areas and by isolation of a pathogen identified as *M. bovis*.

Simultaneous microbiological examinations excluded the presence of chlamydiae and various bacteria of differential diagnostic importance. In calves of that age, having maternally derived immunity, the pathogenic role of adenoviruses, herpesviruses and parainfluenza viruses can also be ruled out.

Arthritis was accompanied by a pulmonary disease which was similar in nature and course to cases of mycoplasmal pneumonia described earlier by other authors (Gourlay et al., 1976; Langford, 1976; Romváry et al., 1977). Our data are consistent with earlier reports according to which *Mycoplasma bovis* is the pathogen most commonly incriminated in the aetiology of arthropathies.

A new and valuable finding of this study is the demonstration of meningitis as a concomitant of arthritis.

Meningitis associated with lymphocytic infiltration of the meninges and non-purulent perivascular lymphocytic and plasma cell infiltration of the mesencephalon, accompanied by gliosis, has been

observed after experimental reproduction of arthritis caused by *Mycoplasma alcalescens* (Bennett and Jasper, 1978). Our results indicate that meningitis that can be brought into connection with the presence of the pathogen may result from natural infections, too.

The results presented here show good agreement with those of similar investigations conducted abroad also in that the disease was found to cause initially 20%, then 50% or higher calf mortality, depending on the prevalence of infection.

On-the-spot investigations have led us to assume that the source of infection may have been the mycoplasma-contaminated semen. However, lympho-haematogenic transmission of the pathogen from the infected udder to the fetus can neither be ruled out.

Although up to the present the form of mycoplasmosis observed in this study has not represented a widespread problem in Hungarian calf stocks, its high prevalence in affected herds may give rise to substantial losses. In our experience, its polyarthritic form resulting from intrauterine infection causes particularly big losses. This form may occasionally manifest itself also as meningitis and may take a severe course already in the first few days of life.

The presence of mycoplasma-infected cows constitutes a high risk also to the yet uninfected susceptible individuals of the herd. At calving, the infected cows may excrete the pathogen with the mycoplasma-contaminated amniotic fluids. Subsequently, mycoplasmas may be shed in the vaginal and uterine secretions and in the milk for a long time.

As infection is refractory to the usual broad-spectrum antibiotherapy and requires a special, long-lasting and expensive treatment, the losses can be prevented only by removing infected mycoplasma-excretors from the herd. Laboratory examinations are of prime importance in such flocks in order to establish a correct diagnosis which enables efficient and rapid exploration and subsequent elimination of the source of infection.

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PATHOMORPHOLOGICAL AND IMMUNOHISTOLOGICAL CHANGES IN LYMPH NODES OF COWS SPONTANEOUSLY INFECTED BY BOVINE LEUKAEMIA VIRUS

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Prescapular lymph nodes from 109 cows seropositive to bovine leukaemia virus (BLV) were subjected to histologic, cytochemic and in dubious cases also to immunohistologic examination for the presence of light chains of bovine immunoglobulin (Ig) kappa or lambda. By their morphological features, the histologically detected changes were divided into B-zonal hyperplasia (23 cases, 21.1 %), T-zonal hyperplasia (52 cases, 47.7 %), pulp proliferation (6 cases, 5.5 %), hyperplasia of mixed type (18 cases, 16.5 %), and atrophy (10 cases, 9.2 %). Some changes resembled those reported in infections with human or feline immunodeficiency virus. Eosinophilic infiltration was a frequent feature. Immunohistochemical examination revealed only a lambda Ig chain in the cytoplasm of plasma cells or plasmacytoid cells and immunoblasts in pulp proliferation. Cytochemical examination showed a considerable number of cells with a diffuse positive reaction to acid phosphatase (AP).

Key words: Cattle, enzootic bovine leukaemia, lymph nodes, pathology, immunohistology

Bovine leukaemia virus (BLV) is the aetiological agent of chronic lymphatic leukaemia/lymphoma in cows (Burny et al., 1988). The neoplastic form of this disease is of clonal B-cell origin and occurs in less than 10 % of infected cattle (Ferrer et al., 1977; Burny et al., 1980, 1985). Alterations occur most frequently in the lymph nodes, heart, spleen and uterus (Loppnow et al., 1971). Histological examinations suggest the primary initiation of tumorous changes predominantly in the lymph nodes. Considerable follicular hyperplasia and atypical blas-

tic cells in sinuses and in the paracortical zone are frequently observed during the process of tumour development (Ohshima et al., 1982).

Altered non-physiological conditions during the proliferation of B lymphocytes or their progeny can lead, in general, to the development of neoplastic diseases, possibly manifested in an impaired immunoglobulin (Ig) production. This can result in an excessive production of one homogeneous Ig or a portion of an Ig molecule (Boggs and Winkelstein, 1975). Changes in the production of Ig (presence of light-chain restriction) are used in human medicine for their differentiation with regard to reactive (Ig polyclonality) and tumorous (Ig monoclonality) processes (Lennert and Stein, 1981).

This study was aimed at the histological, cytochemical and in the dubious cases also at the immunohistological (presence of light chains kappa or lambda) study of morphological changes occurring in the prescapular lymph nodes of cows spontaneously infected by BLV.

Materials and methods

Animals. Prescapular lymph nodes from 109 cows serologically positive for BLV and from 10 serologically negative cows serving as control were used in the study. The majority of cows belonged to the Black-Spotted and Slovak Spotted breed and were 4 to 6 years old.

Detection of BLV antibodies. An immunodiffusion test (Bioveta Nitra, Czechoslovakia) was used for detecting BLV antibodies in animals.

Cytochemical processing of impression preparations. Dried-up impression preparations were fixed in formalin vapours. Preparations treated in this way were subjected to cytochemical reactions for acid phosphatase (AP), nonspecific esterase (NE) and α-naphthyl acetate esterase (ANAE).

Histological and immunohistochemical examination. The samples were processed in a standard manner, i.e. fixed in 10 % neutral formalin and embedded in paraffin. Sections 5–6 μ in thickness were stained

with haematoxylin–eosin and by Giemsa. Cases selected on the basis of histological analysis were also examined immunohistochemically.

Immunohistochemical reactions were carried out on undigested paraffin sections by means of the peroxidase–antiperoxidase (PAP) method. Briefly, the sections were incubated (18 h at 4 °C) with anti-bovine kappa, anti-bovine lambda sera (Dakopatts, Denmark) diluted 1:1,000 in TBS. They were then incubated with a swine anti-rabbit serum (ÚSOL, CSFR) diluted 1:50 in TBS and finally with a rabbit PAP complex (ÚSOL, CSFR). The immunological reaction was developed with diaminobenzidine or aminoethyl carbazole. Sections were then counterstained with Mayer's haematoxylin.

Evaluation criteria. Preparations were evaluated by optical microscopy at commonly used magnifications. Histological preparations were evaluated by two independent observers. Evaluation was based on criteria used for the examination of human lymph node biopsies.

1. Node architecture – mutual relationship of the B zone, T zone, node pulp, lymph sinuses and mesenchymal framework (node capsule and trabeculae).
2. Status of the follicular apparatus (hyperplasia or follicle atrophy, extinction of follicles) was evaluated in the B zone. Evaluation also included the use of cytological criteria for signs of germinal centre and follicle mantle activation (Plank, 1990).
3. Examination of the T zone consisted of evaluation of the signs of expansion or atrophy, proliferation of postcapillary venules and signs of histiocytic activity.
4. The node pulp was evaluated for its extent and proliferation of plasma cells, immunoblasts and plasmacytoid cells.
5. Sinuses were evaluated for their state of preservation and inactivation, or disappearance of their activation, or possibly for an influx of lymphoid cells into the sinuses.
6. In addition, the entire lymph node was evaluated with regard to the proportion of reactive elements (macrophages, mastocytes).

Results

Macroscopic examination of 109 slaughtered animals did not reveal tumorous changes of the predilective organs. Alterations of some examined prescapular nodes included their moderate enlargement with visible follicular hyperplasia on the cut surface or with a vague structure or possibly cortical atrophy. Histological examinations served as a basis for the determination of pathological features in the lymph nodes (Fig. 1).

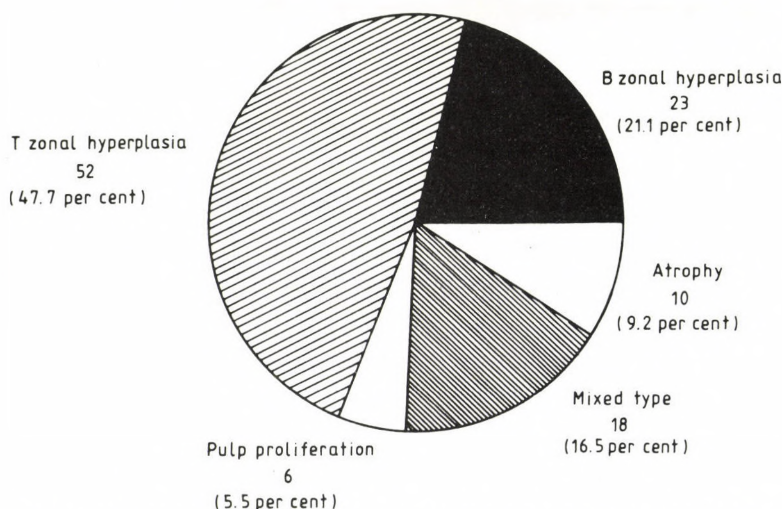
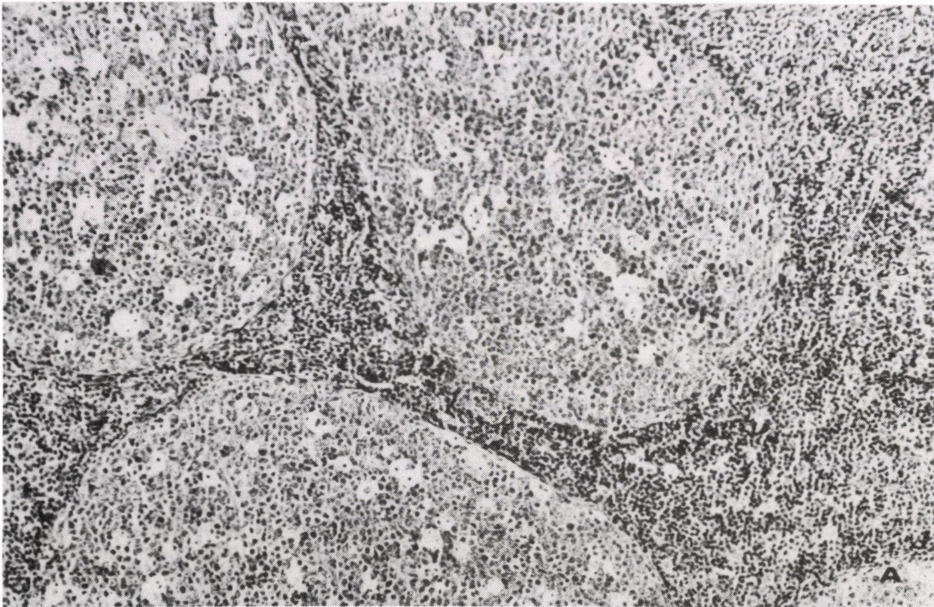


Fig. 1. Pathological features of prescapular lymph nodes in 109 cows

In the case of zonal (follicular) hyperplasia, the follicles were frequently closely packed, sometimes even sharply defined (Fig. 2A). Follicle centres were enlarged, sometimes with ovoid elongation and predominance of centroblasts. Macrophages with a wide light cytoplasm, absorbing debris of disintegrating cells (Fleming's corpuscles), were observed in the follicle centres of some samples (Fig. 2B). In sporadic cases, haemorrhage into germinal nodules and penetration of the follicle germinal centre by lymphocytes were visible.



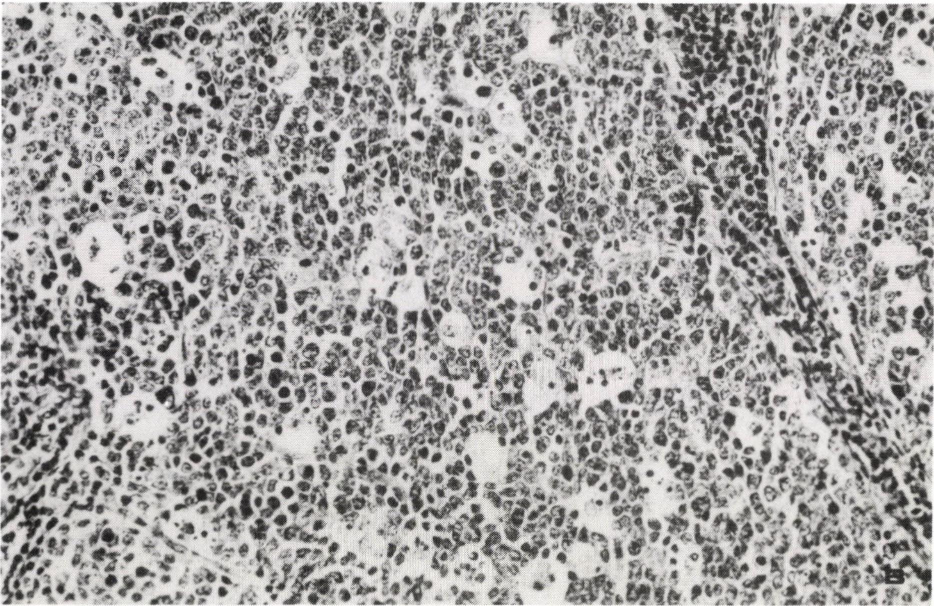


Fig. 2. Sharply defined, hyperplastic germinal centres with tingible body macrophages. Haematoxylin and eosin (HE) (A) x 135, (B) x 270

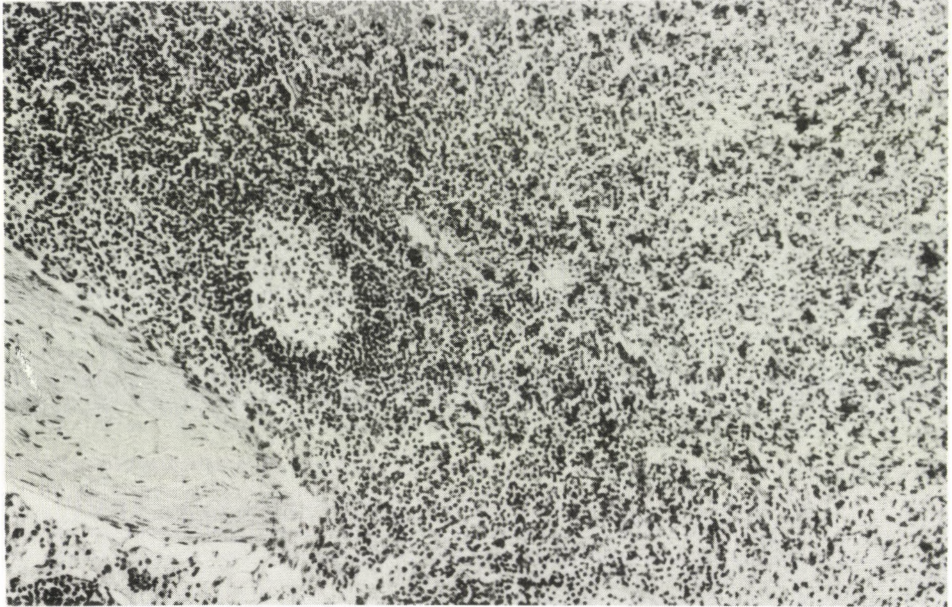


Fig. 3. Extension of T-zonal hyperplasia with follicular involution. HE, x 135

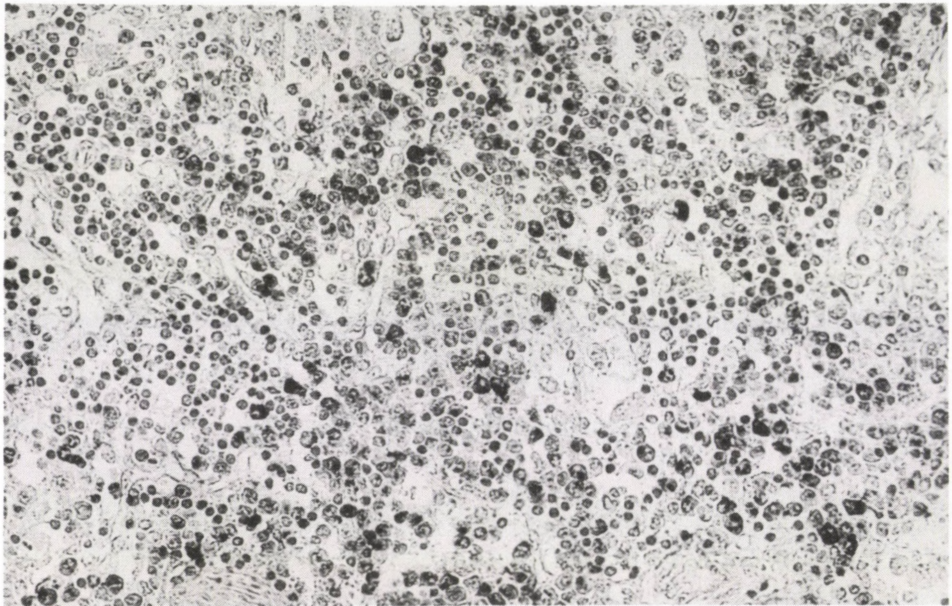


Fig. 4. Pulp proliferation. Immunohistological detection of monoclonal immunoglobulin (lambda chain) in the cytoplasm of plasmocytes. Peroxidase–antiperoxidase, x 270

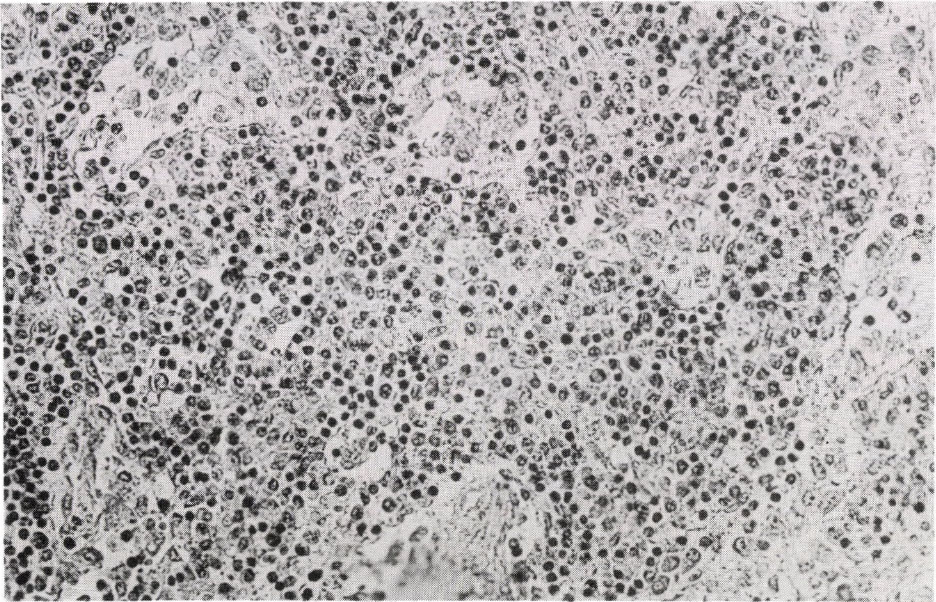


Fig. 5. Pulp proliferation. Plasmocytes are negative for kappa chain of immunoglobulin. Peroxidase–antiperoxidase, x 270

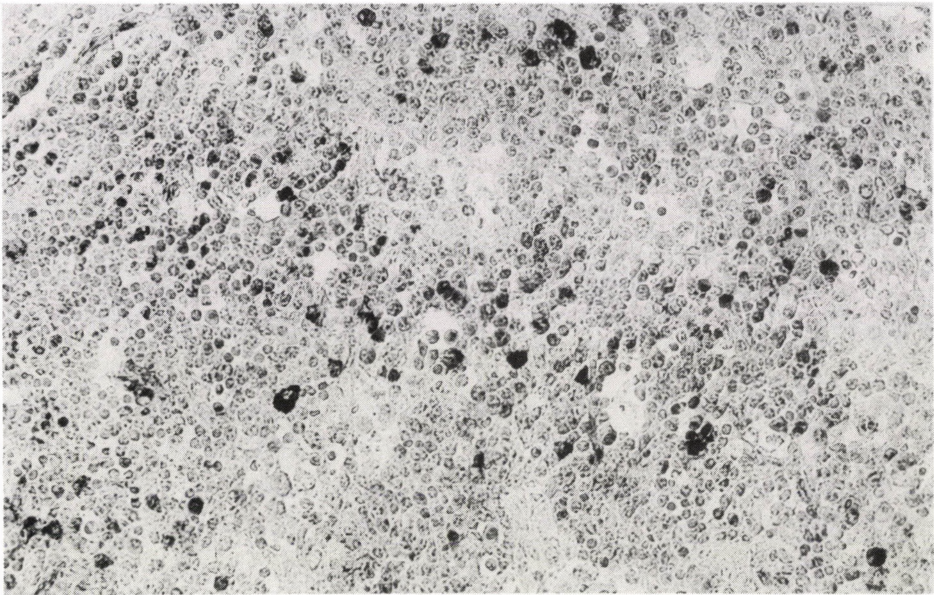


Fig. 6. Control lymph node. Plasmocytes are positive for kappa chain of immunoglobulin. Peroxidase–antiperoxidase, x 270

Prevailing T-zonal hyperplasia consisted of foci of small mature lymphocytes with frequent extension of this zone up to the marginal sinus and in the direction of the hilus (Fig. 3). As a rule, zone activation was accompanied by the multiplication of plasma cells in the pulp. Cytochemical examination showed a predominance of ANAE-positive cells in the impression preparations and, to a small extent, a diffuse positive reaction to AP.

Morphological examination of the six above-mentioned cases showed a pronounced proliferation of plasma cells into the cortex. The cortex was atrophic and pushed in subcapsularly as a result of expansive proliferation of plasma cells. Follicles were small, without signs of activation, with germinal centres indicating a regressive transformation. Similarly, only remnants of the T zone were preserved. Morphologically, this expansive proliferation consisted of proliferation of plasma cells in five cases and of proliferation of cells of plasmacytoid or plasmacytic appearance and less frequent representation of immunoblasts in one case. Only the lambda Ig chain was detected in the cytoplasm of plasma cells, plasmacytoid cells and immunoblasts in all six cases (Figs 4 and 5). Cytochemical examination revealed a considerable number of cells with a diffuse positive reaction to AP.

In 18 cases, morphological examination indicated an increased proliferation of morphologically mature plasma cells. Samples were analysed immunohistochemically. The presence of polyclonal intracytoplasmic Ig (kappa +, lambda +) was observed (Fig. 6.). That is why this proliferation was evaluated as reactive. A cytochemically determined dominance of cells with a diffuse positive reaction to AP was recorded.

Atrophy of the T and B zones was accompanied by a decreased number of lymphocytes in the follicular and extrafollicular zones. Follicles were inconspicuous, depleted, with atrophic centres. Sinus histiocytosis was frequently observed in these processes, pronounced mainly in the marginal sinus (Fig. 7). Proliferation of fibrous ligament tissue was observed in some cases.

Studies of lymph nodes revealed a frequent occurrence of eosinophilic infiltration predominantly in the paracortical region, in the

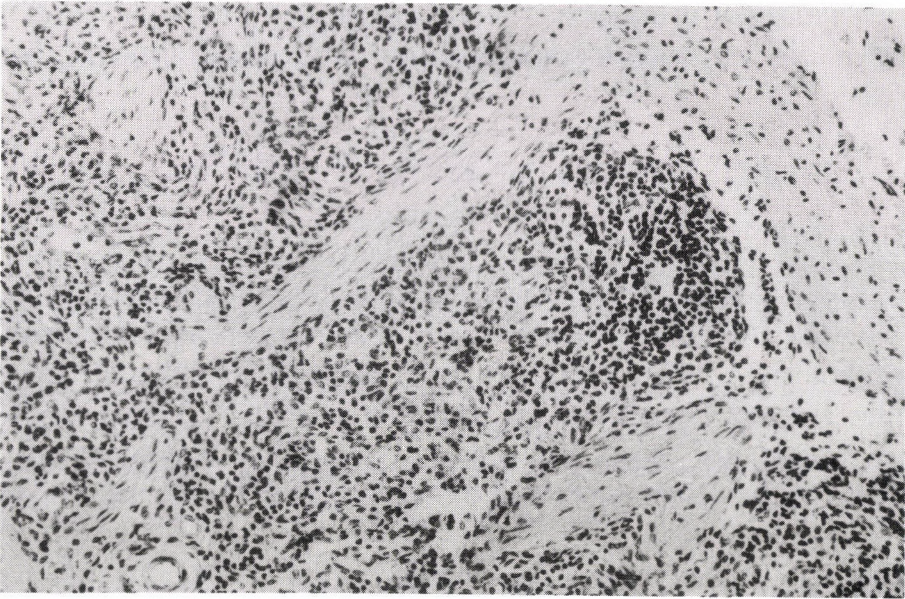


Fig. 7. Connective tissue, vascular skeleton and sinus histiocytosis are prominent in atrophy of lymph nodes. HE, x 135

surroundings of marginal and medullary sinuses. Eosinophilic infiltration was most frequently accompanied by hyperplasia of the paracortical region.

Discussion

Macroscopic and microscopic examinations of lymph nodes from cows spontaneously infected with BLV indicate the development of morphological features indicative of antigenic stimulation. Lymph nodes represent constantly changing and remodelling structures with their histological aspect and cellular composition dependent on the type of the challenge antigen and the course of an immune response (Van den Oord et al., 1986). B cells, accumulated in the follicle centre, where they are subjected to a series of morphological and phenotypic transformations, subsequently leave the follicle and are transformed into immunoblasts under the influence of T-helper cells in the medullary cords (Lennert et al., 1978; Lukes and Collins, 1975).

Follicular hyperplasia observed in this study, characterized by irregular lymphoid follicles, frequent loss of mantle zone, haemorrhage in follicles, and follicular lysis due to gradual infiltration by lymphocytes, was also seen in lymphadenopathies caused by human immunodeficiency (AIDS) virus (Parravicini et al., 1986) and by feline immunodeficiency virus (Brown et al., 1991). The increased phagocytic activity of some follicles is related to the ingested cells which died before or during mitosis (Odartchenko et al., 1967).

The so-called polymorphous pulp hyperplasia (Lennert et al., 1978) is regularly observed in viral infections (Hartsock, 1968). Although this state includes primarily the diffuse hyperplasia of the extranodular element, the T zone can also be affected to a varying extent while follicular hyperplasia is usually inconspicuous (Lennert et al., 1978).

The extranodular composition of lymph nodes plays an important role in the migration of cells and antigens (Balfour et al., 1984), and it is assumed to represent a later stage of the humoral immune response

(Morris et al., 1983). The presence of a considerable number of morphologically mature lymphocytes with the polyclonal Ig phenotype supports this assumption.

The findings obtained for monoclonal cytoplasmic Ig in six cases of pulp hyperplasia are difficult to interpret. From the immunohistological point of view they refer to monoclonal proliferation which occurs simultaneously with the tumorous proliferation. The morphological picture of these cases similarly suggests the presence of an expansive clone of plasma cells, which spreads from the pulp into the node cortex leading to cortical atrophy and impairment of normal node architecture. Their morphological and immunohistochemical appearance thus resembles that of the primary nodal plasmacytomes of human pathology (Lennert, 1976).

At the same time, one must realize that this applies to the isolated enlargement of prescapular nodes without signs of generalization of the tumorous process. These cases can therefore be interpreted as local nodal plasmacytomes, analogically to human pathology, or as a starting monoclonal transformation of plasma cells of the prelymphoma type, not having, however, the character of tumorous proliferations. After all, human pathology, too, recognizes cases of so-called benign monoclonal gammopathy without signs of tumorous diseases (Frizzera, 1985).

The involution of follicles and gradual depletion of lymphocytes with numerous macrophages and sinus histiocytosis are also observed in feline immunodeficiency virus infection (Brown et al., 1991) and in AIDS patients. Some authors classify these alterations as the last stage of morphological changes of lymph nodes in this immunodeficiency infection (Racz et al., 1986; Pallesen et al., 1987).

Eosinophilic infiltration of the paracortical region of lymph nodes in the surroundings of marginal and pulp sinuses was observed predominantly in T-zone hyperplasia. An association between eosinophilia and activated T cells (Colley, 1980; Sanderson et al., 1988) or between the eosinophilic density in lymph nodes and markers of T cell activation (Ben-Izra et al., 1989) has been reported for Hodgkin's disease in humans. Several authors have reported that preclinical EBL

manifestation is detected as peripheral eosinophilia and eosinophilic infiltration (Ohshima, 1982).

These results suggest the dynamic character and diversity of morphological changes occurring in lymph nodes in BLV infections. It can be stated that increased proliferation of mature plasma cells and production of Ig of polyclonal character occur after the antigenic stimulation. However, findings obtained for monoclonal cytoplasmic Ig indicate an alteration of Ig secretion and a possible blockage occurring at a certain stage of cellular maturation.

Further experimental studies on immunologic clonality in relation to the generation of the initial tumorous process could contribute to a better understanding of the development of this tumorous process.

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CONTRIBUTION TO THE PATHOBIOCHEMISTRY OF FURAZOLIDONE-INDUCED OXIDATIVE TOXICITY IN CHICKENS

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Furazolidone (F) and its 5-nitrofuran derivatives, occasionally used in veterinary and human medicine, are active against some microorganisms. This drug is considered to be mutagenic in special bacterial test systems. For public health reasons, the presence of F and other 5-nitrofuran residues in edible mammalian and poultry tissues is strictly prohibited. One-month-old experimental chickens (n=2x25) were fed 300 $mg \cdot kg^{-1}$ furazolidone for 3 days. A separate subgroup (n=11) was used for monitoring the kinetics of F in the blood plasma. Liver, lung and blood plasma samples taken from the experimental and control chickens after treatment were tested for glutathione-peroxidase (GSH-Px), glutathione reductase (GSH-R), catalase (CAT), superoxide dismutase (SOD) activities, reduced and oxidized glutathione (GSH and GSSG), malondialdehyde (MDA), alpha-tocopherol (vE), selenium (Se) and F concentrations. On post-treatment day 2, SOD activity was significantly lowered in the liver only. GSH-Px did not show any characteristic change in any of the tissues. CAT and GSH-R activities were significantly reduced in both organs until post-treatment day 5. At the same time, a significant decrease of GSH accompanied by an increase in GSSG concentration, was found in both tissues. Oral F treatment produced a transient increase in lipid peroxidation levels measured by the formation of MDA. Alpha-tocopherol content significantly decreased in both organs by post-treatment day 2. Se concentrations showed an insignificant rate of decrease. F concentration in the blood plasma reached its peak already 30 min after treatment. In the liver and lungs, F decreased sharply, reaching the detection limit between days 2–5 after treatment. Furazolidone administered *per os* was found to alter the *in vivo* antioxidative enzymatic defense mechanisms. Increased lipid peroxidation and the concomitant oxidative stress may affect the functional integrity of the tissues.

Key words: Furazolidone toxicity, oxidative enzymes, malondialdehyde, tocopherol, selenium

Furazolidone [N-(5-nitro-2-furfurylidene)-3-amino-oxazolidone], a synthetic 5-nitrofuran derivative, is a yellow, odourless, crystalline powder of bitter after-taste. It is practically insoluble in water, ethanol and some organic solvents. Together with other 5-nitrofuran derivatives occasionally used in veterinary and human medicine, F is active against a wide range of bacteria. It also serves as a therapeutic aid for preventing coccidiosis in chickens. Both the antimicrobial activity and the animal toxicity of nitrofurans depend on the presence of a nitro-group (O_2N^-) located at the 5-position of the furan ring (Botsoglou et al., 1989).

The small intestine serves as the first site of furazolidone metabolism *in vivo*. This function is not dependent on enteric bacteria but can be attributed to xanthine oxidase activity located in the cells of the intestinal mucosa. This compound will undergo further metabolism in the liver and kidney under both aerobic and anaerobic conditions. Specific reduced metabolites have been detected and identified in the urine of both conventional and germ-free animals (Hoogenboom et al., 1992).

The acute toxicity of 5-nitrofurans in rodent species is characterized by irritability, seizures, tremor and death from respiratory failure. *Per os* administration of furazolidone to rats results in testicular atrophy with inhibited spermatogenesis and hypertrophy of both the seminal vesicle and the adrenal gland (Kari et al., 1989).

Furazolidone seems to be mutagenic in a variety of bacterial test systems. This compound can induce gene mutations without the activation of the S-9 microsomal fraction in both *Salmonella typhimurium* and *Escherichia coli*. It has been demonstrated that the above-mentioned eukaryotic subcellular preparations can convert some 5-nitrofurans into mutagenic metabolites. It seems to be noteworthy that furazolidone metabolites have been shown to bind covalently to proteins and nucleic acids (DNA, RNA). Evidence has been obtained that furazolidone is carcinogenic to the rat mammary gland. These data suggest that human exposure to furazolidone via the consumption of animal foods might somehow be involved, through the chemically induced toxicity, in the aetiology of cancer (Kari et al., 1989).

In vivo studies have shown that redox-cycling of the furazolidone molecule is a metabolic factor of decisive importance. Under anaerobic conditions this may proceed via one-electron reduction of the nitro-group to a nitro-anion radical, catalysed primarily by cytochrome P_{450} reductase and other cytosolic enzymes. In the presence of molecular oxygen the previously formed nitro-anion radical will be auto-oxidized, regenerating the parent compound and forming superoxide anions. The reactive anion may be dismutated either spontaneously or through the presence of superoxide dismutase, forming hydrogen peroxide. Via the iron-catalysed Haber-Weiss reaction, this could lead to the formation of highly reactive hydroxyl radicals. By peroxidating and destabilizing the sensitive subcellular membranes, the reactive oxygen species are thought to exert deleterious effects on the critical cell functions (Martin, 1983).

With a view to their role in *in vivo* enzymatic defence mechanisms, we decided to determine certain factors of the free-radical scavenging antioxidant system: superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px), glutathione reductase (GSH-R), alpha-tocopherol (vE), malondialdehyde (MDA), protein content, glutathione (GSH) and selenium concentration in selected tissues or in the blood plasma of the experimental chickens.

Materials and methods

Birds. Both the experimental and the control group included 25 one-month-old male Tetra SL broiler chickens (body mass: 587 ± 74 g). For determining furazolidone concentration in the blood plasma just after the treatment (within 5 h) a separate group (n=11) was formed. These birds were housed in metal wire cages, with free access to mixed experimental chicken feed and tap-water. The birds were kept at room temperature (20–22 °C) and were exposed to alternate cycles of 12 h light and darkness. The experimental chickens were treated with 300 mg·kg^{-1} furazolidone thoroughly mixed in the diet fed

ad libitum, for 3 days. The control chickens consumed an unsupplemented ration (Table 1).

Experimental design and sample preparation. After oral treatment with furazolidone, the chickens of both groups were exsanguinated on days 0, 1, 2, 5, 10 and 17 after treatment. Blood, liver and lung samples were taken immediately after decapitation. The organs were weighed and rinsed in ice-cold saline to remove excess blood. The blood plasma and tissue samples were kept in refrigerator at –20 °C until analysed. Heparinized whole blood was prepared and used for immediate determination of enzyme activities.

The moderately thawed samples were finely minced and homogenized with Ultra-Turrax (Janke-Kunkel, KG) in a sufficient volume of ice-cold 0.01 M potassium phosphate buffer (pH 7.4) to produce a 20% homogenate. This was centrifuged at 10^4 *g* for 10 min in a refrigerated K-26D (Janetzki) centrifuge. The post-mitochondrial supernatant was decanted and recentrifuged at 105,000 *g* for 60 min in a refrigerated Beckman L7-65 ultracentrifuge equipped with a 50.2 Ti rotor to obtain microsomal fractions. For the preparation of samples for malondialdehyde (MDA) determination the homogenizing medium contained 3.0 mM EDTA as protective chelator.

Enzyme activity determinations. Superoxide dismutase (SOD) activity in the homogenates was measured by Marklund's (1976) method of inhibition of pyrogallol (1,2,3-benzentriol) auto-oxidation. Catalase (CAT) activity was determined spectrophotometrically by hydrogen peroxide (H_2O_2) disappearance at 240 nm as described by Lück (1963). Glutathione peroxidase (GSH-Px) activity was determined spectrophotometrically in the homogenates, using cumene hydroperoxide as substrate, according to Blanchflover et al. (1986). Glutathione reductase (GSH-R) activity was assayed as the decrease in absorbance at 340 nm and expressed as μmol NADPH oxidized/g tissue homogenate/min according to the method of Clemens and Anderson (1980).

After n-hexane (UV-grade) extraction, solid-phase column clean-up (C_{18}) and mixing with 60% sulphuric acid to avoid retinol interference, alpha-tocopherol content of the tissues was measured

spectrofluorimetrically against the blank at 298 nm excitation and 330 nm emission by the modified method of Razagui et al. (1992).

Table 1

Composition of the diet

Ingredients (%)	
Yellow corn	53.50
Soybean meal (48.0% crude protein)	37.50
Animal fat	5.00
Dicalcium phosphate	1.75
Limestone	1.25
Salt	0.50
DL-methionine	0.30
Vitamin mix[1]	0.25
Trace mineral mix[2]	0.05
Calculated composition	
Crude protein, %	18.60
Metabolizable energy, MJ/kg	12.10
Calcium, %	0.70
Phosphorus, available, %	0.65
Linolic acid, %	1.00
Furazolidone[3], mg/kg	300.00

[1]Provides per kilogram of diet: 6,000 IU vitamin A, 750 IU vitamin D_3, 15.00 mg tocopherol, 4.0 mg riboflavin, 10.0 mg Ca-pantothenate, 30.0 mg nicotinic acid, 300.0 mg choline chloride, 3.0 mg vitamin B_6, 0.010 mg vitamin B_{12}, 0.70 mg folic acid, 0.08 mg d-biotin, 2.00 mg menadione (as MSBC), 2.2 mg thiamine (as thiamine mononitrate);
[2]Provides as parts per million of diet: 8.0 ppm Cu, 80.0 ppm Fe, 0.35 ppm I, 60.0 ppm Mn, 40.0 ppm Zn, 0.150 $mg \cdot kg^{-1}$ Se (actual analysis);
[3]Exclusively in the feed of the experimental group

For determination of the thiobarbituric acid (TBA) reacting lipid peroxidation product (malondialdehyde, MDA), the blended tissue was mixed with 5% trichloroacetic acid, n-butanol (UV-grade), and centrifuged (10^4 g, 10 min, +4 °C) to get a clear supernatant. 0.2% TBA in water was added to the extract. It was subsequently placed into a boiling water-bath for 30 min in scaled tubes. After cooling, 1 ml of 1.0 M phosphate buffer (pH 7.0) was added. Subsequently, the

samples were applied to a C_{18} reverse-phase cartridge (Analytchem International) which had previously been washed with 5 ml methanol followed by two washes with 5 ml of distilled water. After applying the sample, the cartridges were rinsed twice with 5 ml of water to elute the interfering substances not bound to the C_{18} support. The bound fraction was than eluted from the cartridge with 5 ml of methanol. This eluate was then evaporated to dryness under N_2. The dried sample was taken up in 250 μl of water, centrifuged and filtered through a disposable 0.45 filter (Acro, LC3A, Gelman). 100 μl of clear supernatant was then injected into the HPLC instrument. HPLC analysis (on a Pye Unicam-4002 video liquid chromatograph with PU-4015 Quaternary Solvent Delivery System, PU-4021 Diode Array Detector and PU-4850 Video Chromatography Control Centre) was carried out using a C_{18} reverse-phase column (Partisil-100 DS, 10 μm, 25 cm x 4.6 mm I.D.). The column was eluted isocratically at room temperature with 10% acetonitrile, 0.6% tetrahydrofurane in 5.0 mM phosphate buffer (pH 8.0) at a flow rate of 1.0 ml/min. The eluate was monitored at 523 mm using 0.032 absorbance units on the full scale according to Squires (1990).

The protein content of the ultracentrifuged tissue supernatant (10^5 g for 45 min) was estimated by the method of Lowry et al. (1951).

The 1-fluoro-2,4-dinitrobenzene (DNP) derivative of the reduced glutathione (GSH) and oxidized glutathione (GSSG) content of the above-mentioned subcellular fraction was determined the HPLC method of Reed et al. (1980). A reverse-phase ion-exchange Waters μ-Bondapak NH_2-column (30 cm x 3.9 mm) with a bonded surface coating of 3-amino-propyl groups was used for the analysis. The GSH-DNP derivative was detected at 350 nm. For the isocratic elution, *solvent A* (methanol:water=4:1, v/v) and *solvent B* (272 g sodium acetate trihydrate, 122 ml deionized water and 378 g glacial acetic acid) were prepared. To obtain the working solvent, these solvents were mixed as follows: 200 ml of solvent B added to 800 ml of solvent A. For this analysis, the above-mentioned chromatographic system was used.

The high-sensitivity determination of selenium in chicken consisted of the following steps:

(1) Dry-ashing at 450 °C, with addition of magnesium nitrate solution to prevent the elemental loss. The ash content was taken up quantitatively in 0.5 M nitric acid solution. Subsequently, the nitrous gases were completely evaporated (Vidáné Poroszlay and Sas, 1988).

(2) The selenium content of the analyte was reduced in hydrochloric acid solution by the addition of sodium tetrahydroborate to hydrogen selenide in a continuous borohydride generator (PU-9360). The analysis was carried out in air/acetylene flame in PU-9400 type atomic absorption spectrometer. Its burner was equipped with a UNICAM Data Coded Selenium-Hollow Cathode Lamp and slotted tube atomic trap (STAT) quartz tube to increase analytical sensitivity. The instrument was tuned for selenium determination by the following parameters: wavelength: 196.1 nm, lamp current: 10 mA, slit: 1.0 nm, integration time: 5 sec and double beam mode (Norheim and Haugen, 1986).

For furazolidone preparation, liver and lung samples were homogenized (IKA-Turrax) with 1.5 M KH_2PO_4 containing 0.2% sodium azide. For extraction, acetonitrile was added to the homogenate which was then centrifuged at 4,500 *g* for 10 min. The supernatant was extracted with dichloromethane : ethylacetate (1:1, v/v) and centrifuged as above. The upper organic layer was removed, evaporated to dryness at 45 °C under a gentle stream of nitrogen. Subsequently, n-hexane and phosphate buffer (0.67 M, pH 5.0) were added to the dry residue, mixed, centrifuged, and 100 μl of the aqueous phase was subjected to HPLC. Separations were carried out with a Spherisorb 5 Cyano (5 μm) column (50 x 2.1 mm) fitted in front of the analytical column. The eluent included the solution of 0.01 M sodium acetate buffer (pH 5.0) and methanol (6:4, v/v). Flow rate was 1.50 ml/min. The analyses were performed at room temperature at 365 nm with a detector setting of 0.001 AUFS. The calibration graph covered the 0.5–50 ng/g concentration range obtained for spiked samples (Laurensen and Nouws, 1989). For this analysis, the previously described HPLC system was used.

Results

To assess the possible importance of changes of the *in vivo* antioxidant system during furazolidone toxicity, the effects of this orally administered drug on the superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px) and glutathione reductase (GSH-R) activities of chicken liver and lungs were determined. The activities of SOD (Fig. 1A) in the liver were significantly lowered on day 2 after treatment but no such tendency could be observed for the lungs. GSH-Px activity (Fig. 1C) did not show any significant changes in any of the tissues examined.

In contrast, CAT activities were significantly decreased in both organs on post-treatment days 1, 2 and 5, and returned to the control levels by day 10 of the experiment (Fig. 1B). Similarly, the changes in GSH-R activities (Fig. 1D) were similar to those observed for CAT activity. It should be noted that changes in the related protein concentrations of microsomal fractions of the two organs could not account for the changes observed in enzyme activities, as no significant difference could be demonstrated in total protein concentrations of the organs between the furazolidone-treated and the control chickens throughout the experimental period (data not shown).

Since depletion of glutathione has been suggested as a mechanism whereby some xenobiotics and other chemicals initiate cell injury (Perchellet et al., 1986; Subrahmanyam et al., 1987; Sun, 1990), levels of both reduced (GSH) and oxidized glutathione (GSSG) in the liver and lungs of furazolidone-treated and control chickens were measured also in the present study. Figs 2A and 2B show the concentrations of GSH (A) and GSSG (B) in liver and lung microsomal fractions of the above-mentioned groups. In control chickens, the respective concentrations of GSH and GSSG remained relatively constant throughout the experimental period. Approximately 90–95% of glutathione was present in the reduced form (GSH). At the same time, oral treatment of the birds with furazolidone resulted in a significant decrease in the GSH concentrations of both organs on days 2 and 5 of treatment. This phenomenon was accompanied by a concomitant

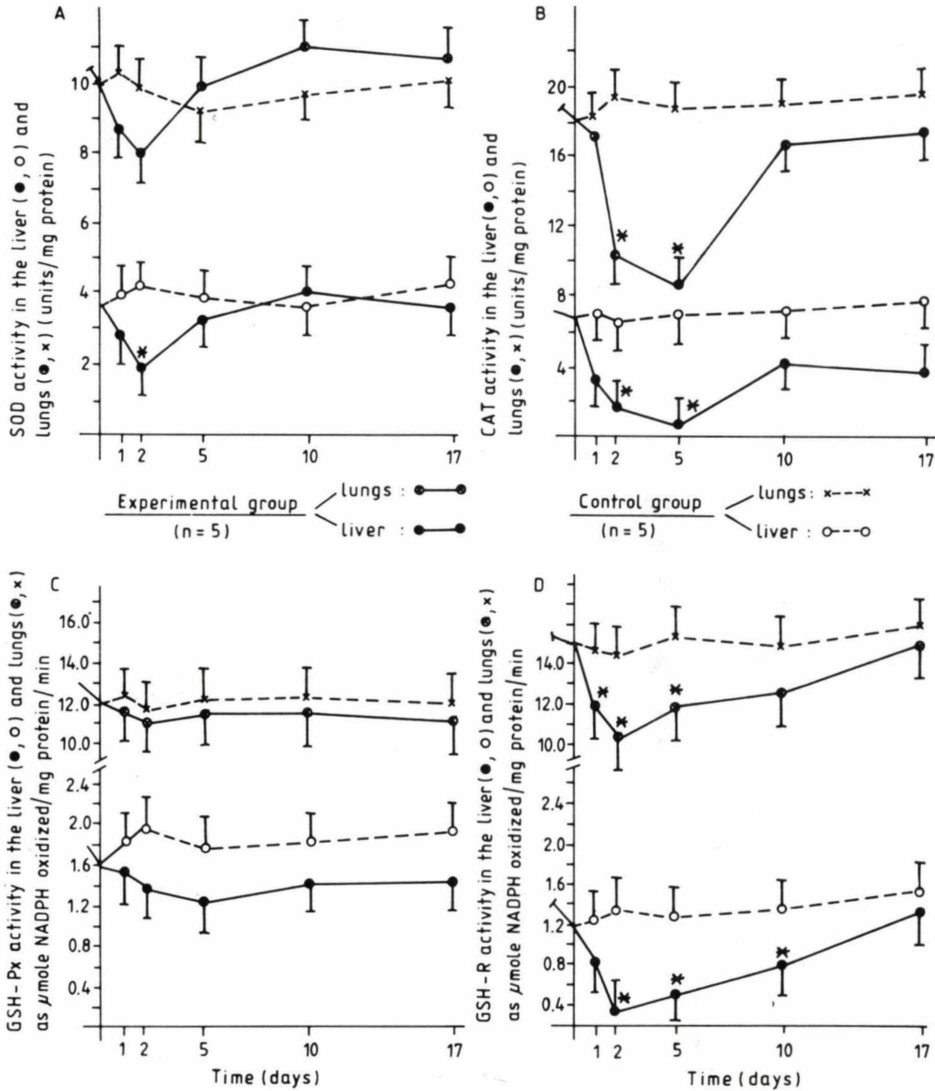

Fig. 1. Oxidative enzyme activities in the liver and lungs of chickens orally treated with furazolidone (300 mg·kg^{-1}) and of untreated (control) chickens. A: Superoxide dismutase (SOD); B: Catalase (CAT); C: Glutathione peroxidase (GSH-Px); D: Glutathione reductase (GSH-R). Each value represents the mean ± SEM for 5 birds. Asterisks (x) indicate a statistically significant difference ($p<0.05$) between experimental and control birds

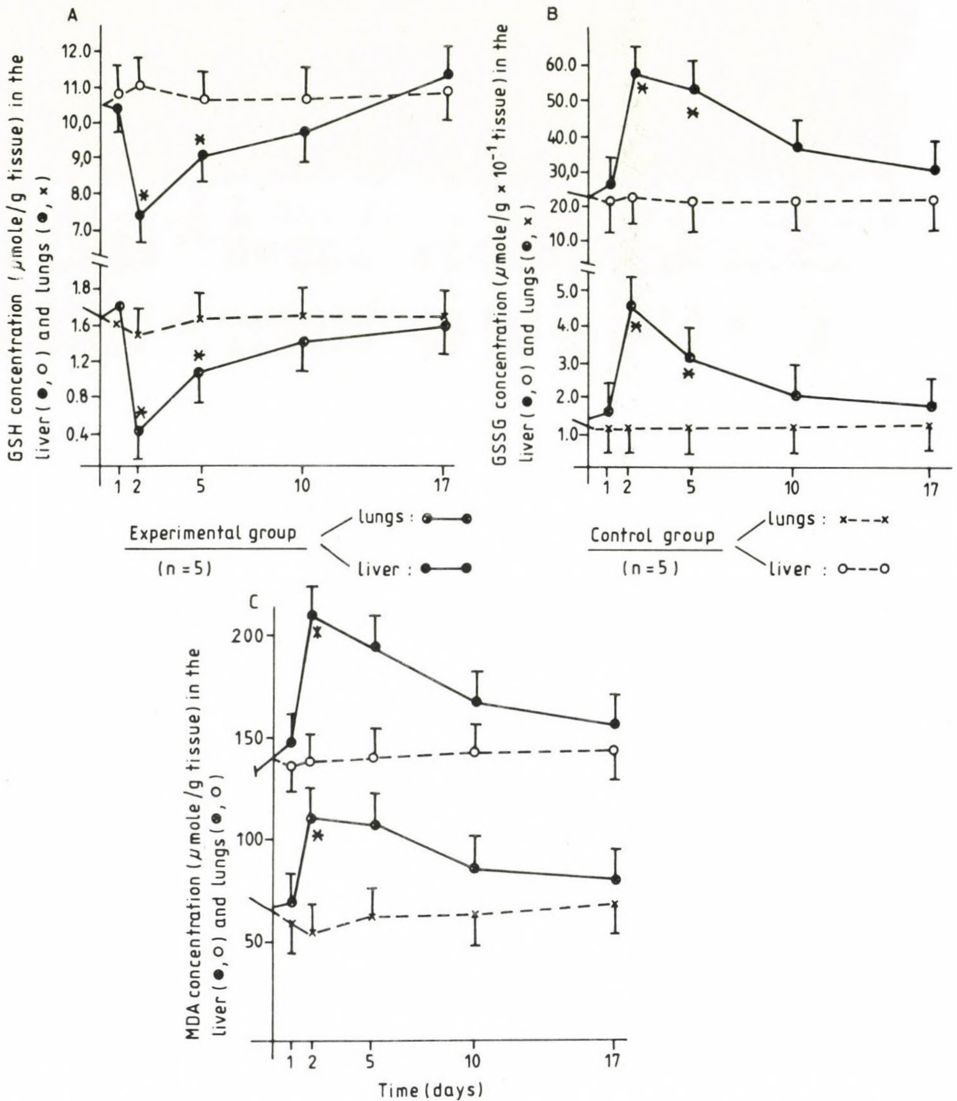

Fig. 2. Liver and pulmonary redox substrates and thiobarbituric acid-reactive compound(s) in chickens orally treated with furazolidone (300 mg·kg^{-1}) and in untreated (control) chickens. A: Glutathione (GSH); B: Oxidized glutathione (GSSG); C: Malondialdehyde (MDA). Each value represents the mean ± SEM for 5 birds. Asterisks (x) indicate a statistically significant difference ($p<0.05$) between experimental and control birds

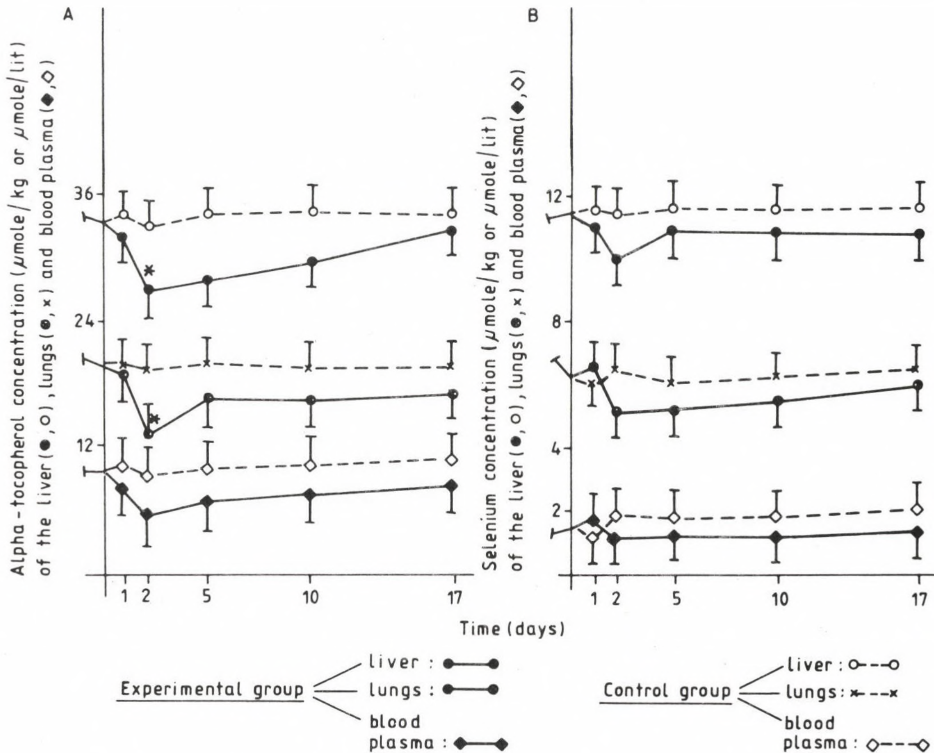

Fig. 3. Alpha-tocopherol and selenium concentration of the liver, lungs and blood plasma of chickens orally treated with furazolidone (300 $mg \cdot kg^{-1}$) and of untreated (control) chickens. A: Alpha-tocopherol; B: Selenium. Each value represents the mean ± SEM for 5 birds. Asterisks (x) indicate a statistically significant difference ($p<0.05$) between experimental and control birds. (Conversion factors: Alpha-tocopherol μmole/kg or /lit. x 0.431 = μg/g or μg/ml. Selenium μmole/kg or /lit. x 0.0789 = μg/g or μg/ml)

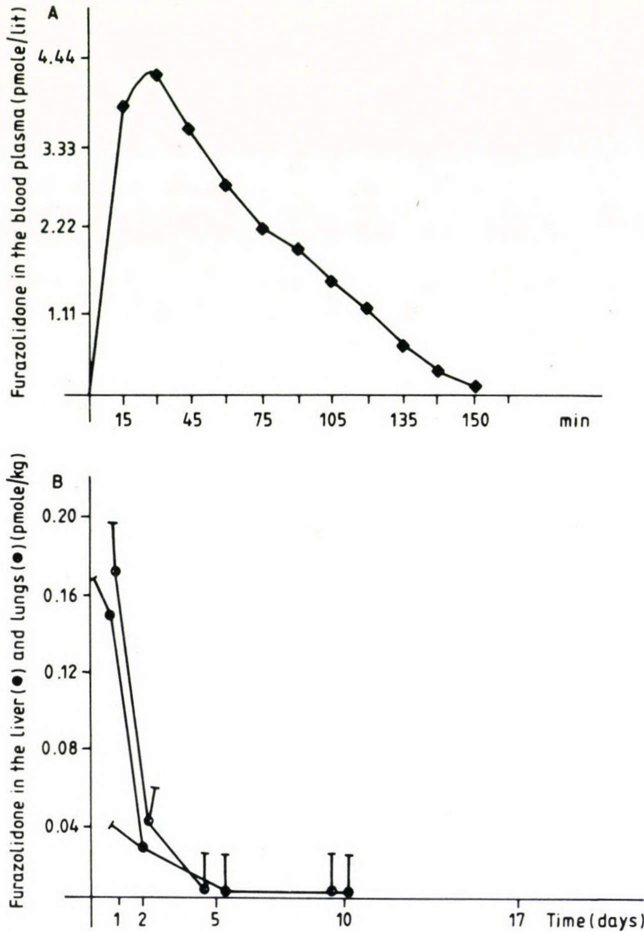

Fig. 4. Furazolidone levels in the blood plasma (A), liver and lungs (B) of chickens after oral administration (300 mg·kg^{-1}). Each value represents the mean ± SEM for 5 birds. Asterisks (x) indicate a statistically significant difference ($p<0.05$) between experimental and control birds. (Conversion factor: furazolidone pmole/lit. or /kg x 450.4505 = ng/ml or ng/g)

increase in GSSG concentrations of both organs. These data suggest that reduced glutathione was oxidized after the administration of furazolidone.

Previous studies have shown that peroxidation of subcellular membrane lipids is a possible mechanism of acute oxidative stress, the related induction of mutagenesis, carcinogenesis and subsequent lethal injury (Cheeseman et al., 1986; Masotti et al., 1988). In this experiment, the levels of lipid peroxidation in homogenates of both organs from control and furazolidone-treated birds were also measured. Fig. 2C shows that oral furazolidone treatment produced a transient increase in lipid peroxidation levels as measured by the formation of malondialdehyde (MDA) as thiobarbituric acid reactant. The control level of MDA in the lungs was half that of the liver. A furazolidone-treatment-related maximum (statistically significant) increase in MDA content of both organs was observed on day 2 after oral treatment. MDA concentrations of both liver and lungs decreased to the control level by post-treatment day 10. The applied thiobarbituric acid reactant method measures the later phases of lipid peroxidation (Horton and Fairhurst, 1987).

Vitamin E, as an antioxidant, acts by scavenging free radicals. As an integral part of cell membrane structures, in helps in the maintenance of their integrity. *In vivo* this function could hinder the peroxidation of unsaturated membrane lipids (see Fig. 2C) and the generation of reactive free-radicals (Cynamon et al., 1985). As a consequence of oral furazolidone treatment, the alpha-tocopherol content had significantly decreased in both the liver and the lungs by post-treatment day 2. Subsequently, the concentration of this vitamin isomer gradually increased, mainly in the liver, until it reached the control level. Parallel to these changes in the tissues, alpha-tocopherol in the blood plasma showed an only slight, insignificant decreasing tendency until day 2 of the experiment (Fig. 3A).

As far as the selenium concentrations of the tissues and body fluids are concerned, only an insignificant rate of decrease was found in the liver and kidney, and a practically unaltered content of this

element was observed in the blood plasma up to post-treatment day 2 (Fig. 3B).

After oral furazolidone treatment had been completed, the concentration changes of this drug in chicken blood plasma (sampled at 30-min intervals) and in the liver and lungs (daily sampling schedule) were analysed. Furazolidone content of the blood plasma reached its peak concentration already 30 min after treatment and 120 min later it dropped below 0.15 pmole/l (Fig. 4A).

Furazolidone concentration of both the liver and lungs decreased sharply and between post-treatment days 2 and 5 it reached the detection limit (Fig. 4B).

Discussion

The results of the present study show that an orally administered single toxic dose of furazolidone alters the *in vivo* enzymatic defence mechanisms and subsequently it affects the functional integrity of some tissues (liver and lungs).

The mechanism(s) by which furazolidone causes tissue injury is (are) not clearly understood. The results presented in this paper support the hypothesis that furazolidone activates, at least in part, some factors of the oxidative stress mechanisms: increased lipid peroxidation and decreased GSH/GSSG ratio are both sensitive indicators of biochemical consequences resulting from oxidative stress (Sun, 1990; Yamazaki, 1987). These results are consistent with the known toxicological properties of nitrofurantoin (Sorrentino et al., 1987). Nitrofurantoin-induced lung damage was enhanced in chickens exposed to vitamin E deficient diets and in those with selenium deficiency (Peterson et al., 1982). It should be clarified whether the reactive oxygen species were due to the direct action of nitrofurane application or to the immigration of inflammatory neutrophils in response to a tissue damage induced by the above drug. During phagocytosis, these cells can generate and secrete large quantities of reactive superoxides into the extracellular space, causing parenchymal

cell injury (Witz et al., 1985). The maintenance of the *in vivo* integrity of the organism and of the single cells in oxidative stress is known to depend on the balance between reactive radical generation and activity of the defence system against it. Their imbalance leads to health consequences when increased reactive radical production overwhelms the capacity of the free radical scavenging defence systems for detoxifying the increased flux of superoxide radicals (Selvam and Anuradha, 1988). Similar results have been reported by other researches, showing that the toxicity of the tested chemicals is related to alteration of redox-cycling in animals and humans: namely, depleted GSH, GSH-peroxidase, tocopherols, selenium and elevated activity of superoxide dismutase and glucose-6-phosphate dehydrogenase (Cagen and Gibson, 1977; Peterson and Eaton, 1989; Peterson et al. 1982).

The present results suggest that the increased intracellular superoxide and peroxide concentration may be partly responsible for the furazolidone-induced tissue damage. A decrease in catalase, GSH-Rase acticities and GSH concentrations would allow significant increases in the tissue's effective superoxide concentrations. Studying furazolidone metabolism and the related oxidative toxicity in isolated rat liver microsomes, Vroomen et al. (1987) suggested that the generated free peroxides play a significant role in enhanced cytotoxicity of the drug applied. Results from *in vitro* studies with isolated pig hepatocytes as well as freshwater fish hepatic microsomes and endothelial cells exposed to furazolidone or nitrofurazone also suggested the generation of reactive peroxides. These findings may have pathologic significance, since oxidative defence mechanisms have been shown to possess protective effects against nitrofurane-induced cytotoxicity and carcinogenicity (Hoogenboom et al., 1992; Washburn and DiGiulio, 1989).

The role of intracellular glutathione (GSH) in the detoxification of reactive oxygen species has been well established (Shan et al., 1990; Subrahmanyam et al., 1987). GSH is known to serve as a reductant in the metabolism of both hydrogen peroxide and reactive hydroperoxides generated following the peroxidation of membrane lipids, a reaction catalysed by GSH-Rase (Grinblat et al., 1989). During this

reaction, GSH is oxidized to its disulfides; GSSG, the oxidized glutathione is reduced back to GSH by GSH-Rase at the expense of NADPH. The close transient relationship, established in this experiment, between changes in GSH concentrations and GSH-Rase activity would suggest that the decreases in GSH/GSSG ratio appear to be due to decreases in GSH-Rase activity. Previous *in vitro* studies with nitrofurane or its metabolites demonstrated the inhibition of GSH-Rase activity by way of direct interference with the catalytic site of the enzyme (Grinblat et al., 1989). GSH depletion was caused by *in situ* nitrofurane perfusion in rat liver isolated hepatocytes and pulmonary endothelial cells (Martin, 1983; Hoener et al., 1989; Rossi et al., 1988).

The present results obtained on the elevated formation of thiobarbituric acid reactive molecular species after oral furazolidone administration call attention to the accelerated process of membrane lipid peroxidation. A moderate correlation was established between nitrofurantoin toxicity and the rate of lipid peroxidation. This toxicity was significantly enhanced in rats fed vitamin E and selenium-deficient diets or exposed to oxygen hyperbaric atmosphere (Boyd et al., 1979).

However, the real mechanism(s) of furazolidone-induced lipid peroxidation observed in the present and in other studies is (are) not known precisely. The tangential interpretation is also supported by results from *in vitro* studies where nitrofurantoin-induced lipid peroxidation was inhibited by ascorbic acid, EDTA, catalase, GSH and superoxide dismutase. These compounds and enzymes could scavenge and/or prevent the generation of reactive oxygen species (Trush et al., 1982). Depletion of GSH observed on day 2 of treatment may have also contributed to the toxicity of furazolidone. It has been reported that decreases in the level of GSSG are followed by extensive increases in lipid peroxidation and total cell necrosis (Hoener et al., 1989). Pathobiochemical alterations caused by lipid peroxidation to the liver and lungs have also been found to occur following exposure of animals to paraquat as redox-cycling agent (Bus et al., 1975; Cagen and Gibson, 1977).

As a consequence of furazolidone treatment, alpha-tocopherol content in the liver and lungs but not in the blood plasma significantly

decreased on post-treatment day 2, in accordance with the increased rate of lipid peroxidation. The selenium content of these samples showed no characteristic change, only a slight decrease. The results of enzymatic and metabolic studies were in good accordance with parallel pharmacokinetic studies of furazolidone in the blood plasma, liver and lungs in this and other experiments (Winterlin et al., 1982).

The results of the present study furnish evidence of furazolidone toxicity induced by oxidative stress mediated mechanisms. These results have provided *in vivo* evidence of lipid peroxidation as a cause of cardiomyopathy induced via a defect of calcium cycling (O'Brien et al., 1991; Reed et al., 1987). The above-listed biochemical factors could account for, and could be used for avoiding, the potential health risks (microbiological resistance, carcinogenicity) of furazolidone and other nitrofurane residues exceeding the maximum tolerance limit (MRL, 5.0 $\mu g \cdot kg^{-1}$) in edible animal tissues and products (Botsoglou et al., 1989; Kari et al., 1989).

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EFFECTS OF CONCURRENT ADMINISTRATION OF LEAD AND SELENIUM ON SOME HAEMATOLOGICAL AND BIOCHEMICAL PARAMETERS OF BROILER CHICKENS

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As compared to intoxication with lead or selenium alone, the concurrent administration of lead and selenium to broiler chickens produced more deleterious effects characterized by adverse changes in haematological parameters and severe alterations in serum total protein, aspartate aminotransferase (AST), cholesterol and alkaline phosphatase levels. Deleterious effects were more pronounced when lead was administered as second toxic chemical in the presence of selenium in the diet of the birds. Significant haematological depressions leading to anaemia were recorded during subacute lead toxicosis when selenium was concurrently administered in the feed, indicating an enhancement of lead toxicity in the presence of selenium.

Key words: Lead, selenium, concurrent administration, haematological parameters, biochemical variables, chicken

The interaction of lead and selenium has been studied by some authors in rats (Cerklewski and Forbes, 1976), dairy calves (Neathery et al., 1987), ducklings (Goldman and Dillon, 1982), quails (Stone and Soares, 1976) and hens (Madej et al., 1988), but not in broiler chickens. Various authors have reported an enhancement of toxic effects when these two metals were administered concurrently.

The experiments were performed in the framework of project 4.100.201

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However, a mild protective effect of selenium during lead toxicosis has also been reported in ducklings (Goldman and Dillon, 1982) and rats (Cerklewski and Forbes, 1976). It is not clear from the above papers what amount of selenium or what ratio of selenium to lead concentration of the body tissues is responsible for the ameliorative or enhanced toxic effects observed during concurrent administration of these two elements. Furthermore, in all of the above-mentioned experiments selenium and lead were administered simultaneously. This might result in masking of the effects of one toxic substance by the other one at a higher level of toxicity. Selenium and vitamin E have a very similar metabolic function in the prevention of *in vivo* peroxidation of fatty acids (Chow and Tappel, 1974; Turner and Finch, 1991). However, the toxicological interaction of lead and selenium has not been studied yet.

In the light of the above facts, this project was designed to adapt broiler chickens to the lowest toxic levels of one metal and, by their subsequent exposure to the second toxic element, to study alterations occurring in the metabolism and toxicity of the other metal.

Materials and methods

Experiment 1

Broiler chickens (Astra B) were kept on basal feed (BK-2 broiler grower mash) for two weeks. Subsequently, the birds were divided into nine groups. Six of the groups were given diets supplemented with selenium (15 mg per kg feed) as sodium selenite (Na_2SeO_3), selenium plus vitamin E (15 and 200 mg per kg feed) as sodium selenite and alpha tocopherol, or lead (1,200 mg per kg feed) as lead acetate [$Pb(C_2H_3O_2) \times 3H_2O$]. These diets were administered throughout the experiment. Three groups were kept on basal feed without addition of any toxic substance. After an adaptation period of 12 days, the second toxic substance (selenium or lead) was administered as a single oral dose (8 or 1,200 mg/kg body mass, respectively) to induce acute

toxicosis of the substance in broiler chickens of the different groups. In this way, birds of groups SL, SEL and LS were concurrently administered selenium and lead at a different ratio. Birds of groups SO, SEO and CS received selenium while those in groups LO and CL received lead only. Chickens of group CO were kept as control. The design of the experiment is shown in Table 1.

Three birds from each group were sacrificed at 24 h post-intoxication (PI) to collect blood and serum. The remaining birds were observed for up to two weeks when all survivors were killed, and blood and serum samples were collected.

Experiment 2

In experiment 2, the grouping of birds and administration of the first toxic substance in the feed were essentially the same as in experiment 1 (Table 1). The adaptation period, however, was extended to four weeks, and the second toxic substance was administered to different groups on alternate days for four weeks at the following doses: selenium at 1 mg and lead at 400 mg per kg body mass. Three birds were sacrificed from each group at week 1, 2 and 4 PI to collect blood and serum samples.

Haematological and biochemical methods

Erythrocyte and leukocyte counts (using a haemocytometer), haemoglobin (Drabkin method) and packed cell volume (by the micro-haematocrit technique) were determined as described by Coles (1986).

Serum was used to determine alanine aminotransferase (ALT) and aspartate aminotransferase (AST) by the method of Reitman and Frankle (Lachma, N. P. Brno Chempol, Czechoslovakia), alkaline phosphatase (AP) by the method of Bassey and Lowery (Biochem. POCH, Gliwice, Poland), total protein by the biuret method (Biochem.

POCH, Gliwice, Poland), and cholesterol by a colorimetric method (Biochem. POCH, Gliwice, Poland).

Table 1
Design of the experiment

a. First experiment

Groups	No. of birds	1st toxic substance in feed (mg/kg feed)	Adaptation period (days)	2nd toxic substance, single oral dose (mg/kg body mass)
SO	13	selenium (15)	12	nil
SL	13	selenium (15)	12	lead (2000)
SEO	13	selenium (15) plus vitamin E (200)	12	nil
SEL	13	selenium (15) plus vitamin E (200)	12	lead (2000)
LO	13	lead (1200)	12	nil
LS	13	lead (1200)	12	selenium (8)
CS	13	nil	12	selenium (8)
CL	13	nil	12	lead (2000)
CO	13	nil	12	nil

b. Second experiment

The design of the second experiment was essentially the same as that of the first experiment, with the following differences: (1) each group comprised 15 birds; (2) the adaptation period was four weeks; (3) the second toxic chemical was administered orally on alternate days for four weeks after the adaptation period at the following dose levels: selenium 1 mg/kg body mass, lead 400 mg/kg body mass

Comparisons were made between the groups which received the same chemical as a second toxic substance (lead or selenium toxicosis) and their counterparts which received a single or a non-toxic substance in the feed. The data were evaluated by analysis of variance and the means of different groups were compared by Duncan's multiple range test at the probability level of 0.05 (Steel and Torrie, 1960).

Results

Lead toxicosis

In experiment 1 (acute toxicosis), packed cell volume was significantly higher in all groups but CL than in the control group at 24 h PI. The haemoglobin level and erythrocyte counts of group SL were lower than those of the control group (CO) at 24 h and at 2 weeks PI (Table 2).

Table 2

Comparison of the haematological parameters of different groups of chickens in acute lead toxicosis (mean ± SD)

Group (dietary addition)	Lead* mg	Packed cell volume per cent	Haemoglobin g/l	Erythrocytes 10^{12}/l	Leukocytes 10^{9}/l
		at 24 h PI			
SO(Se)	0	33.7±2.1[b]	93.6±08.0[c]	3.09±0.8[c]	18.7±2.0[abc]
SL(Se)	2000	32.7±0.5[b]	69.6±29.0[a]	1.81±1.0[a]	25.7±1.2[cd]
SEO(Se+E)**	0	32.3±1.5[b]	92.6±10.0[bc]	2.80±0.4[bc]	15.2±2.1[b]
SEL(Se+E)	2000	34.7±2.3[b]	94.0±12.0[b]	2.46±0.2[abc]	18.5±4.3[ab]
CL(-)	2000	28.0±3.5[a]	68.3±14.0[a]	2.12±0.4[ab]	28.5±10.0[d]
CO(-)	0	28.0±1.0[a]	78.3±30.0[ab]	2.26±0.9[abc]	20.6±6.5[ab]
		at two weeks PI			
SO(Se)	0	24.3±0.6[a]	73.6±20.0[ab]	2.15±0.2[bc]	18.3±7.7[a]
SL(Se)	2000	23.3±3.8[a]	56.3±10.0[a]	1.54±0.2[a]	22.3±2.5[a]
SEO(Se+E)	0	23.3±2.1[a]	76.3±06.0[ab]	2.10±0.2[bc]	14.8±4.1[a]
SEL(Se+E)	2000	25.3±4.6[a]	62.5±15.0[ab]	1.78±0.4[ab]	40.5±13.4[b]
CL(-)	2000	25.0±5.6[a]	69.3±17.0[ab]	2.63±0.2[c]	22.7±11.7[a]
CO(-)	0	27.3±0.6[a]	78.3±03.0[b]	2.26±0.1[bc]	20.7±6.5[a]

*given per kg body mass orally; **selenium + vitamin E; values in the same column with different superscripts are statistically significant ($P<0.05$)

In experiment 2 (subacute toxicosis), packed cell volume, haemoglobin concentration and erythrocyte counts were significantly lower in groups SL and SEL than in all other groups at week 1 and 2 PI. At four weeks PI, packed cell volume of group SL, haemoglobin level of groups SO, SL, SEO and SEL, and erythrocyte counts of groups SL and SEL were significantly lower than those of groups CL and CO (Table 3).

Table 3

Comparison of the haematological parameters of different groups of chickens in sub-acute lead toxicosis (mean ± SD)

Group (dietary addition)	Lead* mg	Packed cell volume per cent	Haemoglobin g/l	Erythrocytes 10^{12}/l	Leukocytes 10^{9}/l
		at one week PI			
SO(Se)	0	26.3 ± 2.5^{bc}	77.3 ± 09.5^{bc}	2.51 ± 0.2^{c}	21.2 ± 3.3^{ab}
SL(Se)	400	21.7 ± 1.5^{a}	64.3 ± 08.0^{a}	1.60 ± 0.4^{b}	27.0 ± 5.3^{bc}
SEO(Se+E)**	0	25.7 ± 0.6^{bc}	74.3 ± 03.0^{abc}	2.41 ± 0.3^{c}	18.1 ± 2.0^{a}
SEL(Se+E)	400	25.0 ± 1.0^{bc}	67.0 ± 05.0^{ab}	2.07 ± 0.3^{bc}	16.7 ± 2.6^{a}
CL(-)	400	27.7 ± 1.5^{cd}	86.3 ± 08.0^{bc}	2.48 ± 0.2^{c}	29.2 ± 8.5^{c}
CO(-)	0	27.7 ± 1.5^{cd}	86.3 ± 08.0^{bc}	2.48 ± 0.2^{c}	29.2 ± 8.5^{c}
		at two weeks PI			
SO(Se)	0	29.0 ± 1.0^{b}	88.6 ± 07.0^{c}	2.52 ± 0.4^{c}	17.3 ± 1.1^{b}
SL(Se)	400	23.3 ± 2.3^{a}	62.0 ± 03.4^{ab}	1.77 ± 0.2^{b}	22.5 ± 1.0^{bc}
SEO(Se+E)	0	28.0 ± 2.6^{b}	74.0 ± 11.0^{bc}	2.00 ± 0.3^{bc}	11.0 ± 3.9^{a}
SEL(Se+E)	400	23.0 ± 2.0^{a}	50.3 ± 01.0^{a}	1.55 ± 0.3^{b}	26.2 ± 5.0^{c}
CL(-)	400	28.7 ± 1.5^{d}	85.0 ± 16.0^{b}	2.11 ± 0.2^{bc}	16.3 ± 3.5^{ab}
CO(-)	0	29.7 ± 0.6^{b}	88.3 ± 02.0^{b}	2.46 ± 0.2^{c}	20.7 ± 3.8^{bc}
		at four weeks PI			
SO(Se)	0	22.7 ± 2.5^{ab}	64.0 ± 12.0^{a}	1.96 ± 0.7^{bc}	08.7 ± 1.6^{a}
SL(Se)	400	22.0 ± 1.0^{a}	57.0 ± 03.0^{a}	1.46 ± 0.1^{ab}	18.3 ± 1.5^{b}
SEO(Se+E)	0	23.3 ± 0.6^{ab}	66.3 ± 06.0^{a}	1.35 ± 0.6^{a}	28.2 ± 1.9^{c}
SEL(Se+E)	400	23.0 ± 3.0^{ab}	60.0 ± 09.0^{a}	1.16 ± 0.3^{a}	25.5 ± 3.5^{c}
CL(-)	400	27.3 ± 0.6^{c}	83.3 ± 02.0^{b}	2.17 ± 0.5^{c}	18.8 ± 5.5^{b}
CO(-)	0	28.3 ± 0.6^{c}	87.6 ± 06.0^{b}	2.31 ± 0.1^{c}	22.8 ± 3.7^{bc}

*given per kg body mass orally on alternate days; **selenium + vitamin E; values in the same column with different superscripts are statistically significant ($P<0.05$)

No significant differences in ALT and AST levels were recorded at 24 h and 2 weeks PI during acute toxicosis. At 2 weeks PI, total protein levels were significantly elevated in groups SL and SEL compared with groups SO and SEO, respectively. No specific inter-group variations were recorded in cholesterol and AP levels (Table 4).

Table 4

Comparison of biochemical parameters of the blood in different groups of chickens in acute lead toxicosis (mean ± SD)

Group (dietary addition)	Lead* mg	ALT IU/l	AST IU/l	Total protein g/l	Chole-sterol mmol/l	AP IU/l
			at 24 h PI			
SO(Se)	0	1.9±0.0	206±132	27.5±3.8^{ab}	3.4±0.8^{abc}	1063±078^{b}
SL(Se)	2000	5.1±4.3	123±23	24.1±1.0^{a}	4.4±1.0^{c}	311±151^{a}
SEO (Se+E)**	0	3.6±2.6	151±32	29.7±0.9^{b}	3.9±0.4^{bc}	381±140^{a}
SEL (Se+E)	2000	8.2±4.7	203±52	23.9±1.8^{a}	4.1±1.2^{bc}	390±132^{a}
CL(-)	2000	3.5±0.6	146±59	24.0±3.1^{a}	2.9±0.5^{ab}	822±362^{b}
CO(-)	0	3.2±1.1	143±39	35.8±1.0^{bc}	2.3±0.1^{a}	308±036^{a}
			at two weeks PI			
SO(Se)	0	3.5±2.3	162±38	28.9±3.1^{a}	3.7±0.1^{e}	281±114^{a}
SL(Se)	2000	6.9±4.8	271±21	40.0±4.0^{b}	2.3±0.1^{ab}	552±101^{bc}
SEO (Se+E)	0	3.9±2.7	179±22	26.4±0.9^{a}	3.5±0.1^{e}	356±031^{ab}
SEL (Se+E)	2000	1.3±0.5	177±28	40.0±5.3^{b}	2.9±0.4^{c}	387±198^{ab}
CL(-)	2000	2.8±0.0	231±51	37.2±2.4^{b}	2.3±0.1^{ab}	260±087^{a}
CO(-)	0	2.5±0.5	167±21	36.6±0.3^{b}	2.3±0.0^{a}	330±056^{ab}

*given per kg body mass orally one time only; **selenium + vitamin E; ALT: alanine aminotransferase; AST: aspartate aminotransferase; AP: alkaline phosphatase; values in the same column with different superscripts are statistically significant ($P<0.05$)

During subacute toxicosis in the second experiment, AST level was found elevated in all groups as compared to the control at 4 weeks PI. Total protein levels were significantly lower in groups SO and SEO, while those in groups SL and SEL did not differ from the control at 4 weeks PI. Cholesterol and AP levels of groups SL and SEL were also significantly higher than in the control at 4 weeks PI (Table 5).

Table 5

Comparison of biochemical parameters of the blood in different groups of chickens in subacute lead toxicosis (mean ± SD)

Group (dietary addition)	Lead* mg	ALT IU/1	AST IU/1	Total protein g/1	Chole-sterol mmol/1	AP IU/1
			at one week PI			
SO(Se)	0	6.9±2.9[b]	191±52[a]	28.9±2.4[a]	2.9±0.3[ab]	268±096[a]
SL(Se)	400	3.2±3.8[a]	295±15[b]	31.6±3.7[ab]	4.2±0.9[c]	321±057[a]
SEO (Se+E)**	0	8.5±0.8[b]	230±42[ab]	29.6±2.7[a]	2.9±0.1[ab]	441±033[ab]
SEL (Se+E)	400	2.2±2.1[a]	243±53[ab]	38.8±7.2[c]	3.6±0.9[bc]	601±248[b]
CL(-)	400	5.7±3.2[ab]	306±43[b]	43.3±4.2[c]	3.2±0.8[a]	579±055[b]
CO(-)	0	3.5±0.6[a]	184±26[a]	35.6±1.5[ab]	2.2±0.2[a]	280±018[a]
			at two weeks PI			
SO(Se)	0	4.5±2.1	291±71[b]	34.3±1.3[ab]	3.5±0.3[c]	298±09
SL(Se)	400	2.4±2.0	264±12[ab]	29.8±2.7[c]	3.1±0.3[bc]	405±102
SEO (Se+E)**	0	2.5±0.5	223±12[ab]	30.8±0.4[c]	3.4±0.1[c]	367±043
SEL (Se+E)	400	2.5±1.4	264±60[ab]	33.3±1.8[ab]	3.6±0.4[c]	418±055
CL(-)	400	1.3±0.5	245±49[ab]	39.6±3.0[c]	2.4±0.5[b]	356±126
CO(-)	0	3.5±0.6	164±41[a]	37.5±1.3[bc]	2.4±0.1[b]	247±018

Table 5 continued

Group (dietary addition)	Lead* mg	ALT IU/l	AST IU/l	Total protein g/l	Chole-sterol mmol/l	AP IU/l
			at four weeks PI			
SO(Se)	0	3.7±0.9	253±39[b]	29.5±4.7[a]	2.8±0.5[b]	395±077[ab]
SL(Se)	400	5.5±1.4	250±55[b]	35.4±1.3[b]	3.9±0.1[c]	635±148[c]
SEO (Se+E)**	0	5.0±2.0	286±63[b]	28.3±1.2[a]	3.7±0.1[bc]	321±023[a]
SEL (Se+E)	400	3.7±2.8	306±43[b]	37.3±1.1[b]	3.2±0.3[abc]	520±199[bc]
CL(-)	400	6.0±2.1	278±32[b]	32.6±2.1[ab]	3.1±0.3[abc]	318±098[a]
CO(-)	0	2.5±1.1	146±10[a]	36.9±2.6[b]	2.6±1.1[a]	259±031[a]

*given per kg body mass orally on alternate days; **selenium + vitamin E; ALT: alanine aminotransferase; AST: aspartate aminotransferase; AP: alkaline phosphatase; values in the same column with different superscripts are statistically significant ($P<0.05$)

Table 6

Comparison of the haematological parameters of different groups of chickens in acute selenium toxicosis (mean ± SD)

Group (dietary addition)	Selenium* mg	Packed cell volume per cent	Haemoglobin g/l	Erythrocytes 10^{12}/l	Leukocytes 10^{9}/l
			at 24 h PI		
LO(Pb)	0	29.0±2.6	91.0±2.6[cd]	2.55±0.5	20.3±9.8
LS(Pb)	8	28.0±1.7	84.7±6.4[abc]	2.30±0.1	22.7±1.6
CS(-)	8	28.3±2.1	81.7±2.8[ab]	2.35±0.2	21.8±1.5
CO(-)	0	28.0±1.0	78.3±2.9[a]	2.27±0.1	19.0±4.4
			at two weeks PI		
LO(Pb)	0	25.3±3.1	79.3±8.0	2.34±0.1	24.2±5.9
LS(Pb)	8	23.3±3.5	69.7±11.9	2.39±0.4	21.7±1.5
CS(-)	8	ND	ND	ND	ND
CO(-)	0	27.7±1.0	78.3±2.8	2.27±0.1	19.0±4.4

*given per kg body mass orally; ND: not determined; values in the same column with different superscripts are statistically significant ($P<0.05$)

Selenium toxicosis

During acute toxicosis in experiment 1, haemoglobin level showed a significant rise as compared with the control at 24 h PI, while all other parameters remained unchanged throughout the experiment (Table 6). During subacute toxicosis, no difference from the control was found in any parameters but haemoglobin which was significantly lower than in the control group at 4 weeks PI (Table 7).

Table 7

Comparison of the haematological parameters of different groups of chickens in sub-acute selenium toxicosis (mean ± SD)

Group (dietary addition)	Selenium* mg	Packed cell volume per cent	Haemoglobin g/l	Erythrocytes $10^{12}/l$	Leukocytes $10^9/l$
			at one week PI		
LO(Pb)	0	28.7±1.5	93.0±3.6^{c}	3.63±0.3	18.6±2.2
LS(Pb)	1	26.3±1.0	76.7±3.2^{a}	2.22±0.5	22.0±8.0
CS(-)	1	28.0±1.7	84.7±4.0^{b}	2.41±0.5	19.2±2.5
CO(-)	0	29.0±1.0	88.3±2.3^{bc}	2.36±0.1	20.8±1.9
			at two weeks PI		
LO(Pb)	0	27.3±1.5^{ab}	83.3±3.2	2.47±0.5	23.7±2.1^{c}
LS(Pb)	1	26.3±0.6^{a}	87.3±3.2	2.05±0.1	19.3±2.9^{bc}
CS(-)	1	28.3±4.9^{ab}	83.0±6.0	2.41±0.3	15.3±1.3^{a}
CO(-)	1	29.7±0.6^{b}	88.3±2.3	2.46±0.2	20.7±3.8^{bc}
			at four weeks PI		
LO(Pb)	0	25.0±1.0^{a}	78.7±4.9^{a}	2.03±0.2	17.8±1.5
LS(Pb)	1	27.0±1.0^{ab}	78.0±2.6^{a}	2.17±0.1	17.3±2.3
CS(-)	1	25.3±0.6^{ab}	81.0±1.0^{a}	2.13±0.2	20.5±1.0
CO(-)	0	28.3±0.6^{b}	87.7±6.0^{b}	2.31±0.1	22.8±3.7

*given per kg body mass orally on alternate days;
values in the same column with different superscripts are statistically significant ($P<0.05$)

During acute toxicosis in the first experiment, ALT and total protein levels were found decreased in birds of group CS as compared with group CO, whereas the total serum protein level group LS did not differ significantly from the control at 2 weeks PI. At 2 weeks PI, cholesterol was elevated in all groups as compared with the control (Table 8).

Table 8

Comparison of biochemical parameters of the blood in different groups of chickens in subacute lead toxicosis (mean ± SD)

Group (dietary addition)	Selenium* mg	ALT IU/l	AST IU/l	Total protein g/l	Cholesterol mmol/l	AP IU/l
			at 24 h PI			
LO(Pb)	0	6.1±0.7	223±111	40.6±9.1^{c}	4.25±0.6	856±152^{c}
LS(Pb)	8	3.5±1.4	129±026	27.8±0.7^{a}	3.79±0.2	573±230^{b}
CS	8	6.0±1.5	233±109	31.9±4.7^{ab}	4.20±0.8	463±066^{ab}
CO(-)	0	3.2±1.1	143±039	35.8±1.0^{abc}	2.30±0.1	308±035^{ab}
			at two weeks PI			
LO(Pb)	0	6.0±1.5^{c}	396±185	38.0±2.0^{c}	3.33±0.2^{b}	432±162
LS(Pb)	8	4.4±1.4^{bc}	180±079	40.1±3.8^{c}	2.85±0.4^{b}	414±183
CS(-)	8	1.0±0.0^{a}	198±000	27.0±0.0^{a}	2.04±0.0^{b}	414±000
CO(-)	0	2.5±0.5^{ab}	167±021	36.6±0.3^{bc}	2.25±0.0^{a}	330±056

*given per kg body mass orally one time only; ALT: alanine aminotransferase; AST: aspartate aminotransferase; AP: alkaline phosphatase; values in the same column with different superscripts are statistically significant ($P<0.05$)

During subacute selenium toxicosis, AST level was significantly higher in groups LS and CS than in group CO at 4 weeks PI. As compared to the control group, total protein level was significantly lower in group LS but not in groups LO and LS. Cholesterol level was higher in group LS than in the control group at 4 weeks PI (Table 9).

Table 9

Comparison of biochemical parameters of the blood in different groups of chickens in subacute selenium toxicosis (mean ± SD)

Group (dietary addition)	Selenium* mg	ALT IU/l	AST IU/l	Total protein g/l	Cholesterol mmol/l	AP IU/l
			at one week PI			
LO(Pb)	0	5.2 ± 3.6	279 ± 134	44.8 ± 4.5^{c}	3.48 ± 0.3^{b}	860 ± 114^{b}
LS(Pb)	1	3.1 ± 0.6	271 ± 021	38.8 ± 5.0^{ab}	3.10 ± 0.3^{b}	668 ± 116^{b}
CS(-)	1	3.7 ± 1.6	216 ± 044	44.4 ± 1.5^{bc}	3.57 ± 0.3^{ab}	330 ± 172^{a}
CO(-)	0	3.5 ± 0.6	184 ± 026	35.6 ± 1.5^{a}	2.17 ± 0.2^{a}	280 ± 018^{a}
			at two weeks PI			
LO(Pb)	0	3.5 ± 0.6	289 ± 130	37.6 ± 2.3^{bc}	2.73 ± 0.4	418 ± 101^{b}
LS(Pb)	1	3.5 ± 3.5	254 ± 032	34.5 ± 1.8^{b}	2.71 ± 0.3	356 ± 040^{ab}
CS(-)	1	3.1 ± 1.4	209 ± 090	40.7 ± 0.7^{c}	2.98 ± 0.1	111 ± 033^{a}
CO(-)	0	3.5 ± 0.6	164 ± 041	37.5 ± 1.3^{bc}	2.41 ± 0.1	247 ± 018^{ab}
			at four weeks PI			
LO(Pb)	0	6.5 ± 0.8	247 ± 060^{ab}	39.6 ± 3.0^{b}	3.24 ± 0.2^{ab}	1024 ± 391^{b}
LS(Pb)	1	6.1 ± 0.8	292 ± 021^{b}	40.7 ± 2.3^{b}	3.77 ± 0.3^{b}	354 ± 076^{a}
CS(-)	1	3.4 ± 2.0	275 ± 105^{b}	28.7 ± 4.0^{a}	3.39 ± 1.0^{ab}	506 ± 127^{a}
CO(-)	0	2.5 ± 1.1	146 ± 010^{a}	37.0 ± 2.6^{b}	2.12 ± 0.3^{a}	259 ± 030^{a}

*given per kg body mass orally on alternate days; ALT: alanine aminotransferase; AST: aspartate aminotransferase; AP: alkaline phosphatase; values in the same column with different superscripts are statistically significant ($P<0.05$)

Discussion

A variable degree of anaemia has been recorded in different species of animals during lead toxicosis (Morgan et al., 1975; Frarson and Custer, 1982; Carlson and Nielsen, 1985; Pain and Rattner, 1988; Falke and Zwennis, 1990; Yagminas et al., 1990; Patel and Venkatakrishnabhatt, 1992) and selenium toxicosis (Rosenfeld and Beath, 1964; Pekkanen et al., 1987). In the present study, administration of lead for variable periods in the feed (group LO) and as a single or as multiple oral doses (group CL) during experiments 1 and 2 resulted in

haematological parameters which were not different from those of the control group (CO). Similar observations have been recorded during administration of selenium (SO, SEO and CS) in both experiments. The nonsignificant decrease observed in haematological parameters in both lead and selenium toxicosis in the present study could be due to the administration of lower amounts of the substances over a shorter period.

Significant haematological depressions leading to anaemia were recorded during subacute lead toxicosis when selenium was concurrently administered in the feed (groups SL and SEL), indicating an enhancement of lead toxicity in the presence of selenium at the dose levels used in this experiment. Similarly, the increase observed in AP and cholesterol levels in groups SL and SEL as compared to the groups administered selenium only (SO, SEO) or lead only (CL) suggested more deleterious effects exerted on metabolism by concurrent toxicosis caused by the two metals. Hens exposed concurrently to lead and selenium (600 and 1.4 mg/kg, respectively) exhibited more severe depression in packed cell volume and in haemoglobin level than those exposed either to selenium or to lead alone (Madej et al., 1988). Administration of lead and radioactive selenium (Se^{75}) to dairy calves suggested that lead might decrease the absorption of selenium (Neathery et al., 1987). Enhanced deleterious effects caused by the concurrent administration of lead and selenium have been reported from Japanese quails (Stone and Soares, 1976). A nonsignificant change in haematological parameters during acute and subacute selenium toxicosis with or without concurrent administration of lead via the feed of birds (groups LS and CS) suggested that added toxic effects of lead and selenium could be observed only at a certain ratio of the two elements, particularly at high lead and low selenium levels. Administration of selenium (1 mg/litre of drinking water) to ducklings poisoned with lead shots provided partial protection indicated by a lesser increase in thyroid weight as compared to those not given selenium (Goldman and Dillon, 1982). In another study on rats intoxicated with lead, concurrently administered selenium up to a dose of 0.5 mg/kg body mass had a mild protective effect, and urinary

excretion of delta aminolevulinic acid was decreased but higher concentrations of selenium aggravated the toxicity of lead as evidenced by the increased lead levels of the tissues (Cerklewski and Forbes, 1976).

Of the biochemical parameters, the nonsignificant changes found in ALT level in all groups could be explained by Coles (1986). An increase in AST level is indicative of muscular damage which has been reported during selenium toxicosis in hens (Madej et al., 1988), swine (Nebbia et al., 1990) and also in lead toxicosis in calves (Satia et al., 1986) and dogs (Stowe et al., 1973), but not in broiler chickens. A significant decrease in serum total protein level in birds administered selenium only (groups SO, SEO and CS) and its nonsignificant decrease when both selenium and lead were administered to the birds (groups SL, SEL and LS) are indicative of a strong interaction between selenium and lead. However, the mechanism by which the toxic effects of both lead and selenium increase the total serum protein level of birds could not be understood. Concurrent administration of lead and selenium also resulted in more severe regressive pathomorphological alterations in various organs at the light-microscopic and ultrastructural level in broiler chickens (Khan, 1991).

In summary, it can be stated that a concurrent administration of lead and selenium had more deleterious effects on the metabolism of broiler chickens than did either lead or selenium alone.

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BIOCHEMICAL CHANGES OF THE MILK IN EXPERIMENTAL CAPRINE MASTITIS INDUCED BY *MYCOPLASMA* SEROGROUP 11 (2-D)

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Biochemical changes of the milk were studied in 7 lactating goats. The right udder halves of the animals were inoculated through the teat canal with 1 ml of *Mycoplasma* ovine/caprine serogroup 11 (2-D) culture containing $6x10^6$ colony forming units ml^{-1}. The left udder halves of these goats were kept as control. The experiment was continued for 35 days during which period one goat was killed every 5th day. All the goats developed clinical mastitis within 24 h and the infected udder halves remained enlarged for 4–5 days. The milk from these halves was yellow-tinged and showed slight separation of proteins. Subsequently, there was marked reduction in the size and secretion of the infected udder halves, followed by agalactia from post-inoculation (PI) day 22. The mycoplasmas were reisolated from milk samples of the infected halves up to PI day 20. Histopathological examination of these halves revealed changes indicative of acute and chronic mastitis. Biochemical analysis of mastitic milk/mammary secretions showed a marked increase in the concentration of total proteins, immunoglobulins, free fatty acids and phospholipids, while the concentration of total lipids and glycerides initially underwent a considerable decrease as compared to the basal and control values. However, the level of total cholesterol decreased sharply during the initial days while later, from PI day 5, it markedly increased. This study shows that *Mycoplasma* ovine/caprine serogroup 11 is pathogenic to the lactating udder of goats and produces marked biochemical alterations in the milk.

Key words: *Mycoplasma* ovine/caprine serogroup 11, goats, mastitis, total proteins, immunoglobulins, lipids, free fatty acids, phospholipids, cholesterol

Mycoplasma ovine/caprine serogroup 11 was first isolated from natural cases of granular vulvovaginitis in sheep in Australia (Cottew et al., 1974). In India, the same organism was later isolated in our laboratory from natural cases of granular vulvovaginitis in goats (Tiwana et

al., 1984) and from natural caprine mastitis (Prasad et al., 1984). Despite its frequent isolation from caprine mastitis, its role in producing mastitis has remained unknown. The present work was, therefore, undertaken to assess the pathogenicity of *Mycoplasma* serogroup 11 to the lactating udder of goats and to study the biochemical changes developing in the milk after experimental infection through the teat canal.

Materials and methods

Seven lactating goats of a nondescript breed, aged 2 to 3 years, were observed for one week prior to the start of the experiment and were judged healthy and free from subclinical mastitis and any bacterial infection. One ml of a 48-h culture of *Mycoplasma* ovine/caprine serogroup 11 (2-D) of the 3rd passage level, containing $6x10^6$ colony forming units (cfu) ml^{-1}, was inoculated through the teat canal into the right udder halves of all the 7 goats. The left udder halves of these goats served as control and were inoculated with 1 ml of sterile mycoplasma broth. Abnormal changes in the udder, temperature variations and evidence of mastitis (assessed by the California and modified Whiteside mastitis tests) were recorded daily in all the animals. Milk samples were obtained from all the goats before inoculation and then at regular intervals post-inoculation till the onset of agalactia. The milk yield of both udder halves was measured daily. The somatic cell count (SCC) of milk/mammary secretions was also determined. Reisolation of *Mycoplasma* was attempted from the milk samples and from the serum. The pooled milk/mammary secretions were analysed for total proteins (Lowry et al., 1951), total immunoglobulins (Oser, 1965, modified), total lipids (Folch et al., 1957), total phospholipids (Ames, 1965), total free fatty acids (Lowry and Tinsley, 1976), fatty acid composition by gas liquid chromatography (GLC, Luddy et al., 1968), total cholesterol (King and Wootten, 1964) and total glycerides (by difference).

One goat was killed every 5 days up to PI day 35. The udders and their lymph nodes were examined gross as well as histopathologically. For the latter examination, 5 μm thick paraffin sections were cut and stained with haematoxylin and eosin.

Results

The body temperature and appetite of all the goats remained normal throughout the experiment. All the goats developed clinical mastitis of varying degree in their right udder halves from the day after infection. The infected udder halves were hot, painful and enlarged during the first 5 days PI. Thereafter, their size rapidly decreased so that they were very small and more firm in consistency as compared to their uninfected left counterparts at the end of the experiment. There was an about 84 per cent fall in the average milk yield of the infected udder halves within 5 days PI, and 3 goats developed agalactia in their infected udder halves from PI day 22. The mastitic mammary secretion was yellowish-white, thick pus-like and slightly flocculent during the first 4–5 days PI. Later on, it became thin and flocculent. The mastitic milk showed separation of milk proteins which settled down as aggregates leaving a whey-like supernatant fluid when the milk was kept undisturbed. On the other hand, there was no appreciable change in the amount, colour and consistency of the milk drawn from the uninfected udder halves of all the goats. The SCC of milk from the infected udder halves increased markedly from the average basal value of 0.5×10^6 ml^{-1} to 38.3×10^6 ml^{-1} and 49.6×10^6 ml^{-1} on PI day 5 and 8, respectively. Thereafter, these values decreased gradually to reach 8.3×10^6 ml^{-1} on PI day 21, which was still much higher than the preinoculation basal value. The SCC of milk from the uninfected udder halves remained unaffected throughout the experiment.

Mycoplasmas could not be isolated from the preinoculation milk samples of any of the goats. After infection, *Mycoplasma* serogroup 11 was reisolated only from the mammary secretions of the infected udder halves up to PI day 20. It could not be isolated from the serum of in-

fected animals. No bacteria were isolated from the milk of any goat prior to and during the experiment.

Histopathological examination revealed mastitis only in the infected right udder halves. On PI day 5, the infected udder half showed diffuse neutrophil infiltration in the acini and lactiferous ducts, along with vacuolation and exfoliation of the acinar epithelial cells. In the animals killed subsequently, acute mastitis was replaced by chronic mastitis which increased in severity over time after the infection. On PI days 10 and 15, there was severe infiltration by lymphocytes and macrophages, completely replacing the acini in some places, marked fibrous tissue proliferation in the interlobular septa leading to the formation of small pseudolobules while the milk ducts showed chronic galactophoritis. Later on, in the animals killed on PI days 20, 25, 30 and 35, the infected udder halves showed very severe and chronic mastitis characterized by cystic dilatation of a group of acini, lymphoid aggregates adjacent to some lactiferous ducts, fibrosis replacing most of the secretory parenchyma (which was the cause of clinical agalactia), and squamous metaplasia of the epithelium lining the lactiferous ducts. The right supramammary lymph nodes showed reactive lymphadenitis. The uninfected left udder halves and their corresponding lymph nodes did not show any pathological change.

As compared to values of the preinfection and control milk samples, biochemical analysis of pooled mammary secretions from the infected udder halves revealed a marked increase in total protein content, i.e. 4.17 g dl^{-1} and 5.16 g dl^{-1} on PI day 2 and 5, respectively. Thereafter, total protein content decreased gradually (Fig. 1). Total immunoglobulin (Ig) content of the mastitic mammary secretions increased markedly to 0.40 g dl^{-1} on PI day 5. This level was almost maintained till PI day 15, after which it decreased to 0.25 g dl^{-1} on PI day 21 but was still higher than the preinoculation basal value and the control value. However, the total protein and total Ig content of milk from the uninfected left udder halves did not show much change (Fig. 1).

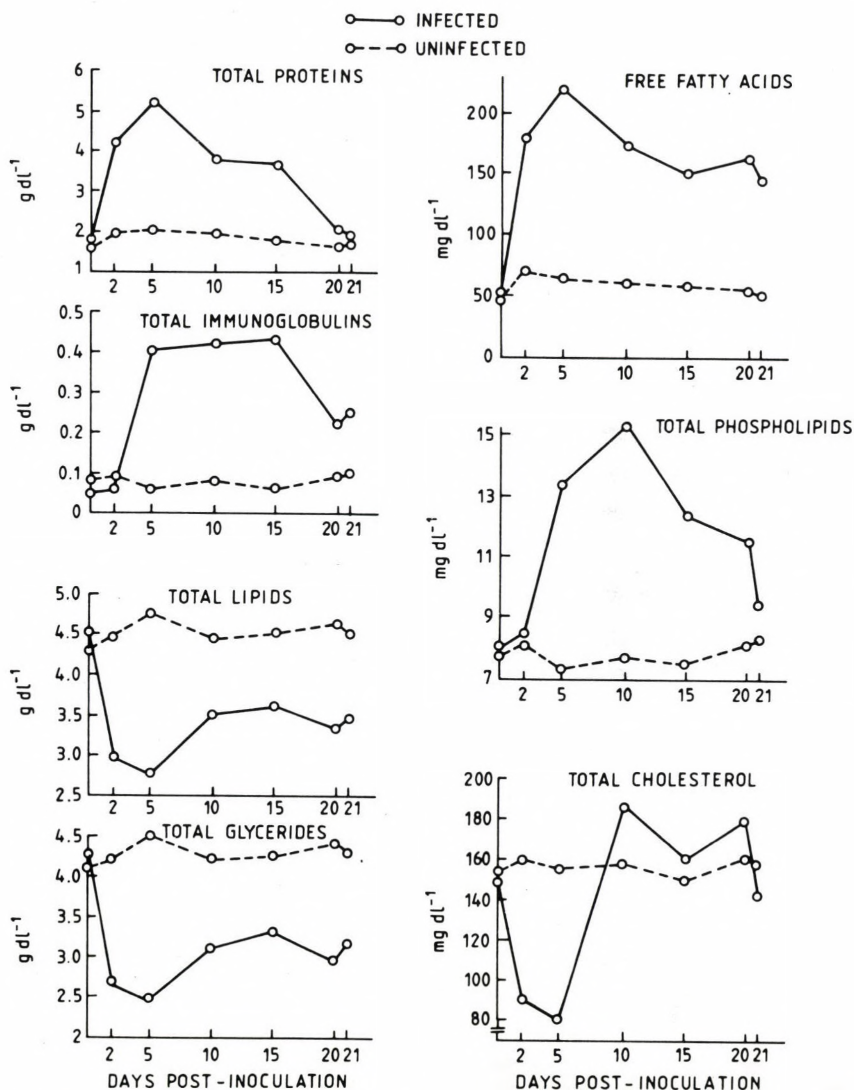
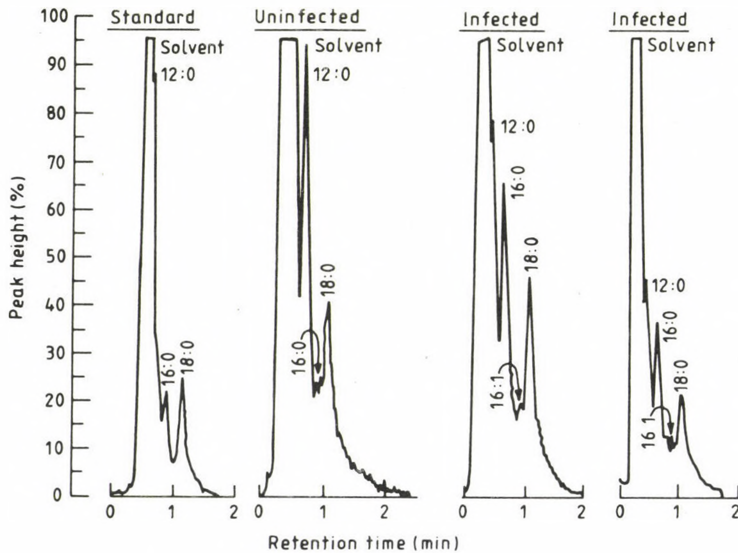


Fig. 1. **Biochemical changes in the milk/mammary secretions of uninfected and infected udder halves of goats after experimental infection with ovine/caprine serogroup 11**

After infection of the right udder halves, the total lipid content of their mammary secretions decreased sharply up to PI day 5, then increased marginally up to PI day 10, and thereafter it was almost constant (Fig. 1). The total glyceride and total cholesterol content of mammary secretions from the infected udder halves showed a similar trend. However, there was no change in the content of total lipids, total glycerides and total cholesterol in the control milk samples (Fig. 1).



Group	$C_{12:0}$	$C_{16:0}$	$C_{16:1}$	$C_{18:0}$
UI	16.90	43.90	5.70	32.50
I	37.43	32.34	4.42	25.90

Fatty acid composition (%)

Fig. 2. Representative GLC patterns of the fatty acid esters from standard and lipids from the mammary secretions of uninfected (UI) and infected (I) udder halves. $C_{12:0}$=myristic acid, $C_{16:0}$=palmitic acid, $C_{16:0}$=palmitoleic acid and $C_{18:0}$=stearic acid

Total phospholipid content of the mastitic mammary secretions increased from 7.92 mg dl^{-1} before inoculation to 13.38 mg dl^{-1} and 15.32 mg dl^{-1} on PI day 5 and 10, respectively. It then declined gradually and reached 9.36 mg dl^{-1} on PI day 21 (Fig. 1). The total free fatty acid content increased sharply from the basal value of 50.72 mg dl^{-1} to levels of 181.24 mg dl^{-1} and 218.47 mg dl^{-1} on PI day 2 and 5, respectively, in the mammary secretions of the infected udder halves. Thereafter, it decreased gradually up to PI day 21 but still remained much higher than the preinoculation basal value. However, total phospholipid and free fatty acid content of the control milk samples remained almost constant (Fig. 1).

Fatty acid analysis of total lipids from the mastitic mammary secretions by GLC (Fig. 2) indicated an increase in the level of myristic acid (C 12:0) and a decrease in the levels of palmitic acid (C 16:0), palmitoleic acid (C 16:1) and stearic acid (C 18:0).

Discussion

The observations of the present study indicate that *Mycoplasma* ovine/caprine serogroup 11 causes mastitis and permanent damage to the secretory epithelium of the milk acini. This damage might be responsible for the marked reduction seen in milk yield and for the subsequent development of agalactia in the infected udder halves. In later stages of experiment, the infected udder halves, which were markedly shrunken and firm, indicated chronic inflammation and almost complete replacement of the secretory parenchyma by fibrosis. In the present study, a remarkable (about 100-fold) increase was observed in the somatic cell count of mastitic mammary secretions. Such, although less expressed, increase in the cell count has also been reported from experimental mycoplasmal mastitis of goats by other workers (Adler et al., 1980; Pal et al., 1983; Prasad et al., 1985; Misri et al., 1988). The continuous reisolation of *Mycoplasma* serogroup 11 from the mastitic mammary secretions up to PI day 20 confirmed that the mastitis was of mycoplasmal origin.

The histopathological findings of the present study were similar to those observed in experimental mycoplasmal mastitis induced in goats with *M. arginini* (Prasad et al., 1985), *M. mycoides* subsp. *capri* (Misri et al., 1988) and in ewes with *M. mycoides* subsp. *mycoides* LC (Banga and Gupta, 1988).

The elevated total protein content in the mammary secretions of the infected udder halves may be due to the passage of serum proteins, especially albumin, into milk as a result of increased capillary permeability due to mastitis (Schalm, 1977). This increase may also be attributed to the higher Ig content and production of larger amounts of membranous material either due to rapidly multiplying *Mycoplasma* and/or to infiltrating leucocytes.

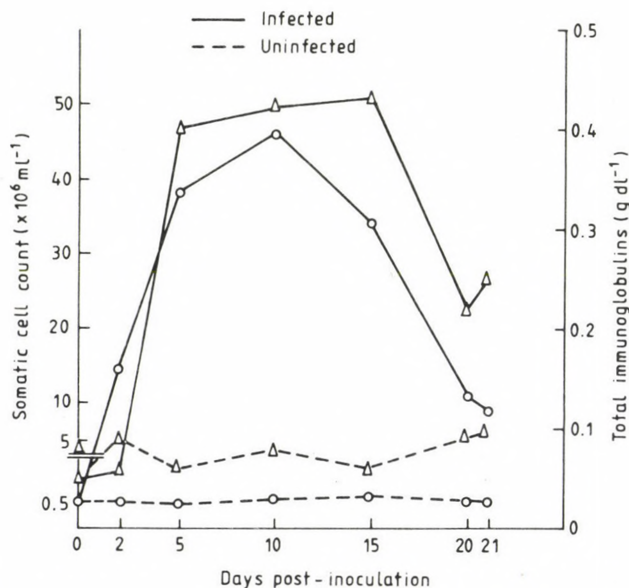


Fig. 3. Corresponding changes in somatic cell count (–o–) and total immunoglobulin level (–Δ–)

A substantial increase in total Ig content may either be due to the humoral immune response and the passage of Ig from blood into mammary secretions as a result of increased vascular permeability (Schalm, 1977) or is attributable to the cell-mediated immune (CMI) response, with local production of Ig by an increased number of infiltrating lymphocytes (Fernald, 1979). The results of the present work indicate that the elevated Ig level is due to the local production of Ig by an increased number of infiltrating leucocytes (Fig. 3) and possibly not due to the humoral immune response. A similar increase in the Ig content of mammary secretions has also been reported in experimental mastitis induced with *M. mycoides* subsp. *mycoides* LC in sheep (Singh, 1988) and with *Acholeplasma laidlawii* in goats (Singh et al., 1991).

The lowered levels of total lipids and total glycerides in mastitic mammary secretions may be due to their utilization after degradation by lipase enzyme secreted or bound to the surface membranes of *Mycoplasma* ovine/caprine serogroup 11. A nonspecific lipase, showing optimum activity in the alkaline pH range, has been demonstrated in some species of mycoplasmas (Smith, 1979). In the present study too, this lipolytic effect of the *Mycoplasma* was confirmed by the elevated level of free fatty acids in the mastitic mammary secretions, particularly during the first 5 days PI (Fig. 1). These free fatty acids and the partial glycerides are possibly utilized by mycoplasmas to meet their energy requirement and/or to synthesize phospholipids for the biogenesis of their plasma membranes. This conclusion was drawn from the presence of much higher levels of phospholipids, especially during the first 10 days PI, in the mastitic mammary secretions. The increase observed in total phospholipid content may be due to the larger amounts of mycoplasmal membranous material, the large numbers of leucocytes released into the milk, and also to the degenerating acinar epithelial cells of the mammary glands. However, the pattern of increase in the levels of phospholipids (Fig. 1) and Ig (Fig. 3), and the number of leucocytes released into mammary secretions (Fig. 3) of the infected udder halves indicate that the initial increase in phospholipids is largely due to leucocytes rather than mycoplasmas. Mycoplasmas, which are organisms rich in cholesterol (Razin, 1978), probably

contribute to this increase after PI day 5, as is evident from the corresponding elevation of the cholesterol level (Fig. 1). Total cholesterol content had a tendency to decrease during the first 5 days PI. Mycoplasmas lack the ability to synthesize cholesterol (Smith, 1979) and are dependent on their environment for the availability of cholesterol, which is incorporated into their plasma membrane (Razin, 1978). The initial decrease in the cholesterol content of mammary secretions may be due to the strong adhesion of mycoplasmas to the mammary acinar epithelial cells (Razin, 1978), along with their continuous multiplication as well as incorporation of milk/cellular membrane cholesterol into their plasma membrane. This needs to be investigated further. The subsequent increase of the cholesterol content (after PI day 5) is probably due to the release of an increased number of cholesterol-rich *Mycoplasma* serogroup 11 organisms into the mastitic mammary secretions. The larger amount of membranous material from leucocytes and degenerating mammary cells rich in cholesterol may also account for the above increase.

The results of fatty acid analysis obtained by GLC suggest that long-chain fatty acids, viz. C 18:0, C 16:0 and C 16:1, are partially degraded to C 12:0 fatty acids. It appears that *Mycoplasma* serogroup 11 partially degrades the long-chain fatty acids of milk to meet its energy needs and elongates medium-chain fatty acids for the biosynthesis of membrane lipids, as mycoplasmas are known to require preformed fatty acids for their growth (Harrison and Lunt, 1980).

The findings of the present study indicate that *Mycoplasma* ovine/caprine serogroup 11 (2-D) is pathogenic to the caprine udder. It produces severe mastitis and causes permanent damage to the secretory parenchyma, leading to a sharp drop in milk yield followed by agalactia. It does not cause any systemic infection in the infected animals. This indicates that the infection remains localized to the inoculated udder half of the animal and produces characteristic physical and biochemical changes in the milk.

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CLINICAL BIOCHEMICAL DETERMINATIONS IN THE MANGALARGA-PAULISTA HORSE: REFERENCE VALUES

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Biochemical values are widely related with environmental agents, sex and age, and are used in disease diagnosis. Numerous reports have been published on the biochemical parameters of different breeds of horses. However, there is a paucity of information concerning Cu-Zn superoxide dismutase (SOD), ceruloplasmin, copper and zinc determinations in the serum. Blood samples from a total of 60 horses of the Mangalarga-Paulista breed, representing three age groups (0 to 4 months old, 6 to 18 months old and adult) were examined. Male horses have a higher mean value of SOD, ceruloplasmin and copper than do females. No significant sex-related difference was observed in serum zinc content of weaned and adult horses. SOD activity was significantly higher in adult animals. Since SOD has a protective effect against superoxide free radical toxicity and possesses anti-inflammatory activity, it is reasonable to assume that the increased activity of this enzyme may be due to an adaptation mechanism which protects the adult animal against oxygen toxicity.

Key words: Blood parameters, horse, Mangalarga-Paulista breed, age-related variations

The normal values of blood constituents have been recorded for various breeds and types of horses. The Mangalarga-Paulista is a race improved by crossbreeding with a Spanish horse population. It became widespread in many countries owing to its diagonal manner of walking that provided security, velocity and stability in the march. The race is fairly unique in that all horses have high endurance and work mainly aerobically. These characteristics permit biochemical adaptations to training (McCully et al., 1991), and the production of large amounts of superoxide radicals by aerobic metabolism (Hass and Massaro, 1987).

Barclay and Hansel (1991) observed that the superoxide radical (O_2^-) appeared to contribute to fatigue in oxidative skeletal muscle. However, neither O_2^- nor other free radicals cause any toxic effect in physiological concentrations. It is obvious that cells must have means of preventing these events. Cu-Zn superoxide dismutase (SOD, E.C. 1.15.1.1.) catalytically scavenges (O_2^-) and has a protective role in cells. Since SOD is an enzyme that carries one copper and one zinc ion at the active center (Novelli et al., 1992), the present paper documents reference values for copper, zinc, ceruloplasmin and Cu-Zn superoxide dismutase in clinically healthy horses of the Mangalarga-Paulista breed. The work presented here was undertaken to determine the effect of sex and age on these biochemical parameters.

Materials and methods

A total of 60 clinically healthy horses of the Mangalarga-Paulista breed were examined. The horses represented three age groups: 10 suckling foals and 10 suckling fillies of 0 to 4 months of age, 10 weaned foals and 10 weaned fillies of 6 to 18 months of age, and 10 stallions and 10 mares.

At the time of blood collection, all horses had minimal to moderate helminth infestation (1,500 strongyle eggs/g of faeces), and had no signs of equine infectious anemia. Horses were maintained on Bermuda grass *(Cynoden dactylon)* pasture with free access to water and salt supplements with measured amounts of alfalfa pellets, oat, and corn ration at 1% of their body mass twice daily.

The components of Bermuda grass were: 39.0% of dry matter, 9.1% of crude protein, 5.2% of digestible protein, 26.0% of crude fibre, 0.49% of calcium, 0.19% of magnesium and 0.27% of phosphorus. The salt offered to horses throughout the study was a mixture of calcium (128.0 mg/kg), magnesium (550.0 mg/kg), phosphorus (66.0 mg/kg), copper (30.0 mg/kg), iron (1.25 mg/kg), methionine (60.0 mg/kg), lysine (20.0 mg/kg), niacine (3.5 mg/kg) and vitamins A (2,000 IU), E (3.8 mg/kg), D (20.0 IU), B (1.7 mg/kg) and K (300 mg/kg). The

vitamin B complex enclosed vitamins B_1, B_2, B_6, B_{12} and pantothenic acid at the same ratio.

The ration fed to weaned and adult horses contained 13.0% moisture, 2.0% ether extracted, 10% vitamins, 1.5% calcium, 0.6% phosphorus and 125.0 mg/kg ethoxiquin. The carbohydrate, crude protein, digestible protein, fibre, minerals, copper and zinc content was 20.8%, 17.0%, 15.6%, 10.0%, 9.5%, 30.0 mg/kg and 180.0 mg/kg, respectively, in the ration fed to weaned animals, and 28.7%, 15.0%, 5.2%, 12.0%, 12.0%, 25.0 mg/kg and 90.0 mg/kg in the ration fed to adult horses throughout the study.

Environmental conditions and pasture composition were maintained constant throughout the study, between October and June. Horses were fasted 3 to 5 h before sample collection. Care was taken to minimize stress. Blood was collected from the jugular vein without anticoagulant. Serum was conspicuously separated within 30 min of collection. The samples were transported to the laboratory in ice box, centrifuged at 3,000 rpm to allow full separation of the serum and stored at 4 °C until used for biochemical analysis.

The serum was used for copper (Nozaki and Zhou, 1987), zinc (Lapugnani et al., 1990) and ceruloplasmin (Henry et al., 1960) determinations. The activity of Cu-Zn superoxide dismutase (SOD, E.C. 1.15.1.1.) was determined in the serum, based on the ability of the enzyme to inhibit the reduction of nitro blue tetrazolium (Sigma) (Otero et al., 1983). Although the two types of dismutases mainly occur in the cytosol or mitochondrial fraction of eukaryotic cells, recently the determination of SOD in serum has been used to investigate its pathophysiological role (Ono et al., 1991).

Values are presented as means ± S.E.M. Significance of difference was tested by Student's *t* test (Zar, 1974).

Results

Superoxide dismutase activity was significantly lower in suckling foals and suckling fillies in relation to the value observed in mares and

stallions. These observations were in accordance with the results obtained for copper, zinc and ceruloplasmin, which were significantly higher in adult animals. No significant alterations were observed in SOD activity and zinc concentrations of weaned and adult animals. There was no significant age difference in ceruloplasmin activity of weaned foals and stallions, and in the copper levels of weaned fillies and mares (Table 1).

The data presented in Table 1 show that stallions have higher serum copper levels than do weaned and suckling foals. Although weaned fillies had a higher mean ceruloplasmin activity than did suckling fillies, the highest ceruloplasmin activity was observed in mares.

Our observations indicate that male horses have a higher mean value of SOD, ceruloplasmin and copper than do females (Table 1). No significant sex difference was observed between weaned and adult animals in serum zinc concentration.

Discussion

Blood parameters are known to vary by age, breed, sex and season. The superoxide radical (O_2^-) is a toxic intermediate in inflammation and carcinogenesis, and is produced by activated phagocytes including monocytes, macrophages and neutrophils, for stimulating osteoclastic bone resorption (Garret et al., 1990). O_2^- releases cycloxigenase products and induces vasoconstriction of rat thoracic aortic rings (Lawson et al., 1990). It may contribute to oxidative skeletal muscle fatigue (Barclay and Hansel, 1991) and small intestinal strangulative obstruction in horses. SOD should be evaluated for clinical use in such cases (Jonston et al., 1989). Statistical comparison of values (Table 1) showed a significantly ($p<0.05$) higher SOD activity in normal adult levels with the corresponding higher copper, zinc and ceruloplasmin concentrations in the serum.

Table 1

Cu-Zn superoxide dismutase (SOD), ceruloplasmin, copper and zinc determinations in the serum of horses

Biochemical parameters	Male			Female		
	Suckling foal	Weaned foal	Stallion	Suckling filly	Weaned filly	Mare
SOD (IU/mg protein)	47.7±3.6*	81.9±7.8[a]*	77.8±5.4[a]*	17.4±5.8*	45.6±7.0[a]*	31.3±6.7[a]*
Ceruloplasmin (U)	209.7±94.2*	258.8±94.9[a]*	281.2±29.2[a]*	47.7±4.5*	50.3±17.8*	211.8±47.7[a]*
Copper (μg/dL)	85.2±36.9*	116.2±48.7[a]*	170.7±64.2[a]*	55.1±13.9*	85.4±36.0[a]*	82.9±7.3[a]*
Zinc (μg/dL)	117.1±13.2*	195.4±16.2[a]*	183.7±19.4[a]	140.3±10.4*	175.6±19.4[a]	170.8±20.1[a]

Values are presented as means ± S.E.M. Significance of difference was tested by Student's *t* test

(*) Values that are significantly different from those observed in the respective male group ($P<0.05$)

([a]) Values that are significantly higher than in suckling animals ($P<0.05$)

The fact that SOD activity was higher in male than in female horses kept on the same diet indicated that increased SOD activity is sex-related and can be due to an enhancement of the adaptation mechanism which protects the male against oxygen toxicity. This observation is in accordance with the finding of Freundt and Ibrahim (1991) that copper and zinc salts showed low or no effects on dismutase activity in rat liver.

Rises in O_2^- induce lipid peroxidation in biological membranes, cause impairment of membrane function (Barber and Bernheim, 1967), and increase nonspecific permeability to ions, eventually causing cell death (Gutteridge and Halliwell, 1988). Lipid peroxidation occurs with age, pigment accumulation and cell loss, which are observed in age-related changes in the brain, retina and skin (Barber, 1966). However, at a physiological level O_2^- does not cause strand breakage or chemical modification of the bases in DNA, or other toxic effects. It is obvious that cells must have means to prevent these events. SOD catalytically scavenges O_2^- and has a cell-protective effect (Chamber et al., 1985).

SOD activity must be maintained at normal values for each age, in view of the deleterious actions mediated by O_2^- in cases when SOD activity is decreased in horses. This would, in turn, permit the clinical administration of SOD as an anti-inflammatory agent or to protect cells against the effects of free radicals. Since the physiological function of SOD is a protective one (White, 1991), it appears that adult horses are exposed to a variety of agents which induce increased O_2^- and, consequently, increase SOD activity as a defence mechanism of the organism (Table 1).

Despite the controversy over the mode of SOD induction, this paper reports the reference parameters for SOD, copper, zinc and ceruloplasmin in the Mangalarga-Paulista horse breed. These values are widely related to the diagnosis of diseases such as inflammation, radiation-induced damage (Yamamoto et al., 1989) carcinogenesis (Eze et al., 1990), aging (Vani et al., 1990), bone resorption (Garret et al., 1990) and oxidative skeletal muscle fatigue (Barclay and Hansel, 1991). We conclude that the normal reference values of SOD, copper,

zinc and ceruloplasmin for the Mangalarga-Paulista horse are related to sex and age, and their alterations can indicate various disorders caused by O_2^-.

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RESTRICTION ENDONUCLEASE ANALYSIS OF HUNGARIAN BOVINE HERPESVIRUS ISOLATES FROM DIFFERENT CLINICAL FORMS OF IBR, IPV AND ENCEPHALITIS

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The DNA of thirty-three Hungarian bovine herpesvirus isolates originating from cattle with various clinical symptoms were compared by restriction endonuclease analysis using *Hin*dIII, *Eco*RI and *Bst*EII enzymes. The *Eco*RI and *Hin*dIII cleavage patterns were similar to those of isolates studied in other countries. Based on the cleavage patterns, the Hungarian isolates could be assorted into groups and subgroups according to the classification system proposed by Metzler et al. (1985, 1986). Based on a new *Bst*EII cleavage pattern observed in group 1, the establishment of two new subgroups, 1a and 1b, were proposed. Three isolates belonged to subgroup 1a, sixteen to 1b, seven to 2a, five to 2b, and one to group 3, which has recently been reclassified as BHV-5. Additionally, one of the isolates showed a mixed cleavage pattern of 1a and 1b. However, no strict correlation was found between the different clinical forms and the established DNA fingerprint groups. There was no evidence of a change in the prevalence of the different genotypes when comparing isolates collected at different times of a 24-year period.

Key words: Restriction endonuclease analysis, bovine herpesvirus, DNA, clinical forms, isolates, Hungary

Bovine herpesvirus 1 (BHV-1) is regarded as one of the major pathogens of cattle. The virus has been associated with clinically, pathologically and epizootiologically different forms of disease. BHV-1 was isolated from infectious bovine rhinotracheitis (IBR), infectious pustular vulvovaginitis (IPV), conjunctivitis, encephalomyelitis, abortion, and generalized systemic disease (Ludwig, 1983).

There have been attempts to differentiate BHV-1 strains isolated from various clinical symptoms based on some of their biological properties. Investigations failed to find differences between IBR and IPV strains in morphology, antigenic relationship or replication in cell cultures.

With the introduction of restriction endonuclease "finger-printing" of the viral DNA, strain differences have been described among the investigated IBR and IPV isolates (Engels et al., 1981). Similar differences were found for other BHV-1 isolates by Misra et al. (1983), Gregersen et al. (1985), Metzler et al. (1985) and Seal et al. (1985). Metzler et al. (1985, 1986) classified the BHV-1 strains into groups and subgroups on the basis of their *Eco*RI and *Hin*dIII restriction cleavage patterns. It was revealed though that the correlation, if any, between the restriction fingerprint patterns and the different clinical forms of disease was not as strict as had been thought initially.

Herpesviruses isolated from bovine encephalitis cases in Australia (French, 1962), in Hungary (Bartha et al., 1969) and in Argentina (Carillo et al., 1983) could be separated from other BHV-1 strains by restriction endonuclease analysis (Brake and Studdert, 1985; Metzler et al., 1986; Magyar et al., 1989), by polyacrylamide gel electrophoresis of their polypetides, and by indirect immunofluorescence using different monoclonal antibodies (Metzler et al., 1986). These viruses, previously designated as BHV-1.3 by Metzler et al. (1986), are now classified as BHV-5 by the Herpesvirus Study Group of the ICTV (Roizman et al., 1992).

The aim of this study was to characterize Hungarian BHV-1 isolates derived from different clinical forms by restriction endonuclease analysis and to compare them with the reference strains.

Materials and methods

Virus strains. The reference strains LA and K22 were kindly supplied by H. Ludwig (Berlin) and strain N569 by A. E. Metzler

(Zurich). For our investigation, all the available Hungarian isolates – previously identified as BHV-1 – were examined. These 33 isolates were collected in the period 1965 through 1989 from cattle with different clinical symptoms at different diagnostic institutes or at the Veterinary Medical Research Institute, Budapest. For DNA analysis, virus isolates with low passage number (below five) were used. The viruses were propagated on monolayers of secondary bovine testicle cells prepared in rolling bottles.

Virus purification and extraction. At 48 h after infection, the culture medium was cleared from cell debris by low-speed centrifugation. The supernatant containing extracellular virus was spun down for 1.5 h at 25,000 rpm (Beckman SW28 rotor). The pellet was resuspended in NTE buffer and sedimented through 30% w/v sucrose cushion for 1.5 h at 24,000 rpm. The virus pellet was resuspended in 2 ml TE buffer and incubated overnight at 37 °C in water bath in the presence of 100 μg/ml Proteinase K (Sigma Chemical Co., U.S.A.) and 0.5% sodium dodecyl sulphate (SDS). The viral DNA was purified by phenol–chloroform extraction and precipitated in a 2.5-fold volume of absolute ethanol in the presence of 0.3 M sodium acetate. The precipitate was pelleted, washed with 70% ethanol, dried and redissolved in TE buffer (Maniatis et al., 1982).

Restriction enzyme analysis. Restriction endonucleases were purchased from Amersham International plc, U.K. (*Eco*RI, *Hin*dIII) and from New England Biolabs, Beverly, MA, U.S.A. (*Bst*EII). Digestions were carried out under the conditions recommended by the suppliers. DNA fragments were electrophoresed through 0.5% agarose gel at 50 volts for 15 h in a submerged gel electrophoresis apparatus. Lambda phage DNA cleaved with *Hin*dIII was used as DNA size marker. The isolates were classified on the basis of their DNA cleavage patterns, by taking into consideration Metzler's classification system on BHV-1 (Metzler et al., 1985, 1986).

Results

For the comparison and classification of the Hungarian BHV-1 isolates, *Hin*dIII, *Eco*RI, and *Bst*EII restriction endonucleases were used as their cleavage sites are mapped on the BHV-1 genome (Mayfield et al., 1983; Engels et al., 1986). In addition, the relatively low number of BHV-1 DNA fragments cleaved with these enzymes was convenient for strain classification.

The different restriction cleavage patterns of the Hungarian herpesvirus isolates originating from different clinical forms obtained with *Hin*dIII, *Eco*RI, and *Bst*EII are shown in Fig. 1. Using *Hin*dIII, four different cleavage patterns could be distinguished. Three different restriction cleavage patterns were found after cleaving the DNA with *Eco*RI and also three in the case of *Bst*EII (Fig. 1).

Nine out of the 38 different *Hin*dIII, *Eco*RI, and *Bst*EII cleavage sites were recognized in the DNA of every isolate, the rest – being located on more variable areas of the genome – existed in certain isolates only. This variation was used as a basis for establishing groups and subgroups. Apart from the variation in the number of restriction cleavage sites on the genome of the investigated isolates, the electrophoretic mobility of certain fragments (especially those that contained parts of the inverted repeat regions of the genome) varied amongst different isolates of the same group. Since this variation did not correlate with other strain differences, it was ignored in the classification.

The Hungarian isolates could be assorted into the three major groups established by Metzler et al. (1985, 1986) after the evaluation of the differences in the *Eco*RI and *Hin*dIII cleavage patterns with the help of published physical maps (Engels et al., 1986). Group 2 had been subdivided into subgroups 2a and 2b (the latter having an extra *Hin*dIII cleavage site) by Metzler et al. (1985). Isolates possessing the characteristics of subgroup 2a or 2b were found during our survey.

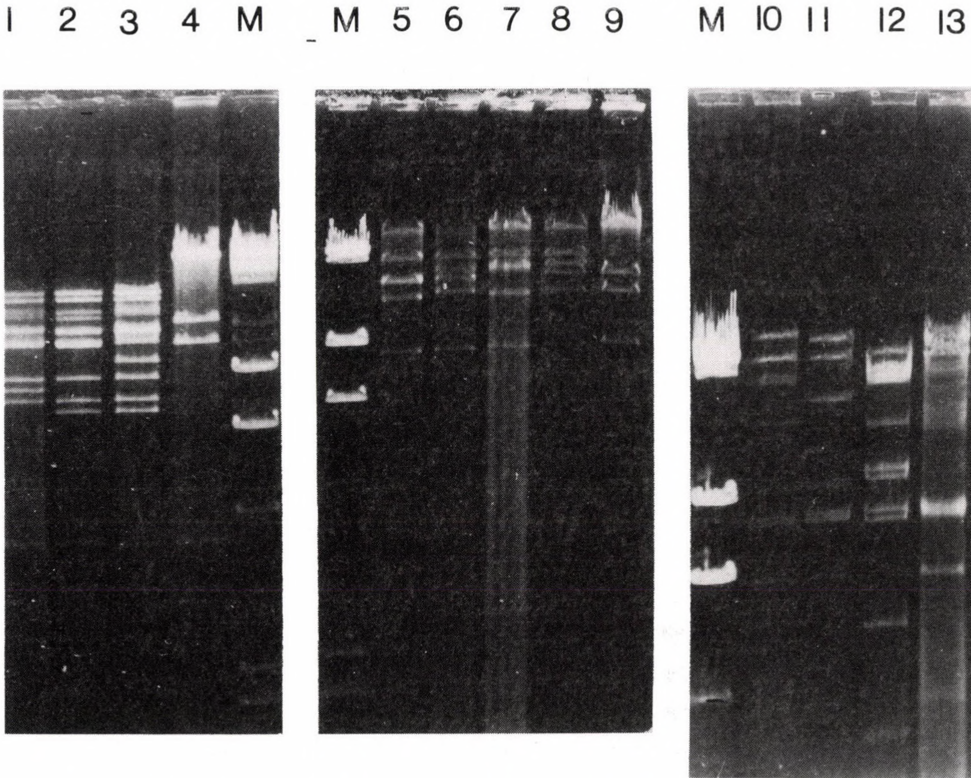

Fig. 1. DNA electrophoresis cleavage pattern of Hungarian BHV-1 isolates belonging to different groups after being cleaved with *Bst*EII, *Eco*RI or *Hin*dIII. Lane M: molecular weight marker lambda phage cleaved with HindIII (fragment sizes in kilobase pairs: 23.1, 9.4, 6.5, 4.2, 2.3, 2.0).
Lane 1-4: *Hin*dIII cleavage patterns of HB178 (lane 1), HB156 (lane 2), HB163 (lane 3) and Na67 (lane 4), representing the characteristic cleavage patterns of groups and subgroups 1, 2a, 2b and 3, respectively. Lane 5-9: *Eco*RI cleavage patterns of HB178 (lane 5), HB162 (lane 6, with different electrophoretic mobility of fragment C compared to lane 5), HB180 (lane 7), HB156 (lane 8, with different electrophoretic mobility of fragment D compared to lane 7) and Na67 (lane 9), representing the characteristic cleavage patterns of groups 1 (lanes 5-6), 2 (lanes 7-8) and 3 (lane 9), respectively. Lane 10-12: *Bst*EII cleavage patterns of HB180 (lane 10), HB178 (lane 11) and Na67 (lane 12), representing cleavage patterns characteristic of subgroup 1a or group 2 (lane 10), subgroup 1b (lane 11) or group 3 (lane 12). Lane 13 represents the *Bst*EII cleavage pattern of the mixed isolate HB183 showing all the fragments characteristic of subgroup 1a and 1b viruses

Table 1

Clinical origin and DNA restriction endonuclease cleavage type of 33 Hungarian isolates after using *Hin*dIII, *Eco*RI or *Bst*EII for characterization

Group	Isolate	Clinical form	Organ	Year
		of isolation		
1a	HB152	IBR	nose	1973
	HB192	IBR	nose	1973
	HB123	IBR	nose	1982
1a+1b	HB183	Enc.	brain	1973
1b	HB153	IBR	nose	1973
	HB195	IBR	lung	1973
	HB196	IBR	lung	1973
	HB107	IBR	nose	1983
	HB159	IBR	lung	1985
	HB144	IBR	nose	1986
	HB179	IBR	lung	1986
	HB197	IBR	nose	1986
	HB150	IBR	nose	1987
	HB151	IBR	nose	1987
	HB161	IBR	lung	1988
	HB162	IBR	lung	1988
	HB177	IBR	nose	1988
	HB178	IBR	nose	1988
	HB182	IBR	nose	1989
	HB194	Conj.	conj.	1975
2a	HB156	IBR	nose	1977
	HB118	IBR	lung	1982
	HB158	IBR	lung	1985
	HB180	IBR	trachea	1987
	HB181	IBR	trachea	1987
	HB124	IPV	vagina	1965
	HB191	IPV	vagina	1965

Table 1 continued

Group	Isolate	Clinical form	Organ	Year
		of isolation		
2b	HB154	Gen.	tonsil	1987
	HB190	IBR	lung	1974
	HB198	IBR	lymph node	1983
	HB160	IBR	nose	1985
	HB163	IBR	nose	1988
3	Na67	Enc.	brain	1967

One isolate showed a mixed restriction cleavage pattern of subgroup 1a and 1b. Isolates within one group were enrolled by their clinical form and isolates with the same clinical form were listed in chronological order of isolation.
Abbreviations: IBR=rhinotracheitis, IPV=pustular vulvovaginitis, Enc.=encephalitis; Conj.=conjunctivitis; Gen.=generalized systemic form of the disease; conj.=conjunctiva

Several Hungarian isolates belonging to group 1 showed a *Bst*EII restriction cleavage pattern (Fig. 1, lane 11) that could not be completely matched to any of the hitherto published *Bst*EII physical maps of BHV-1. With respect to the *Bst*EII physical map of BHV-1.1 (Engels et al., 1986), an additional cleavage site was identified on the inverted repeat regions (estimated map units 0.80 and 0.91, respectively) manifesting in the new cleavage pattern. This allowed us to establish 1b as a new subgroup for these isolates on the analogy of Metzler's subgroups 2a and 2b.

Out of the 33 Hungarian isolates, three belonged to subgroup 1a, sixteen to 1b, seven to 2a, and five to 2b (Table 1). One isolate (Na67) belonged to group 3 (recently reclassified as BHV-5) as previously reported (Magyar et al., 1989) but we failed to find any further isolates belonging to this taxon even from encephalitis cases. One isolate showed the mixed cleavage pattern of groups 1a and 1b when cleaved with *Bst*EII.

The reference strains LA, K22 and N569 showed the characteristics of groups 1a, 2b, and 3, respectively, as previously published (Metzler et al., 1985, 1986).

Discussion

In this work, the available Hungarian isolates previously identified as BHV-1 we examined by restriction endonuclease analysis to study the genetic diversity of this virus in Hungary.

The *Hin*dIII or *Eco*RI restriction cleavage patterns of the Hungarian isolates were similar to the cleavage patterns published for isolates originating from different geographical regions. Therefore, Metzler's classification system could be adopted. As a consequence of an additional *Bst*EII cleavage site on the inverted repeat regions of certain Hungarian isolates belonging to group 1, we suggest that they should be assigned to a new subgroup, 1b.

All major groups and subgroups were represented amongst the Hungarian isolates in the same way as in many other countries in the world, but unlike Australia, where both IBR and IPV isolates belonged to the same subgroup, 2b (Brake and Studdert, 1985), or the United Kingdom, where isolates belonged predominantly to group 1 with some 2b isolates (Edwards et al., 1990). The heterogeneity of Hungarian isolates can easily be explained by the frequent importation of livestock to Hungary from different countries in the past decades. As BHV-1 can establish latency, the introduction of foreign BHV-1 isolates to Hungary with clinically healthy but latently infected animals remains to be a special hazard. As opposed to Australia or the United Kingdom, there is no geographical barrier around Hungary to prevent the introduction of new virus types from neighbouring countries.

We could not find any significant difference in the prevalence of various virus types or clinical forms amongst the isolates originating from different times of a 24 year period. It has to be mentioned though, that the number of isolates connected with pustular vulvovaginitis and especially abortion (no isolate at all) is underrepresented

throughout the whole period when compared to their clinical prevalence in Hungary, even though these forms are not as common as is the respiratory form.

One Hungarian isolate (HB183) showed the characteristics of both groups 1a and 1b when cleaved with *Bst*EII, indicating that a mixed population of viruses possessing two different *Bst*EII cleavage patterns have been isolated. The mixed cleavage pattern of HB183 and that of certain isolates published by others – for example isolates "Grangeneuve" and "Oberhünigen" (Engels et al., 1981) or strain M06 (Gregersen et al., 1985) – indicate, that viruses with different cleavage patterns can be isolated from infected animals at the same time. As there is no indication of a transition of the DNA from one BHV-1 group into another during virus multiplication in one animal, the simultaneous isolation of viruses with different cleavage patterns may either be the result of an infection with mixed virus population or, what is more likely, a new infection may provoke the replication of another virus type which has previously established latency in the animal. This latter hypothesis is supported by the observations made by Whetstone and Miller (1989) during a reinfection experiment.

Table 2

Distribution of the Hungarian BHV-1 and BHV-5 isolates by their clinical forms and DNA types

Clinical origin	total	1a	1b	2a	2b	3
Generalized form	1	-	-	-	1	-
Respiratory form	27	3	15	5	4	-
Conjunctivitis	1	-	1	-	-	-
Genital form (IPV)	2	-	-	2	-	-
Encephalitis	2	1[1]	-	-	-	1
Total	33	3+1[1]	16	7	5	1

[1]The mixed isolate HB183 shared the characteristics of groups 1a and 1b according to the *Bst*EII DNA cleavage pattern

Studying BHV-1 strains from different parts of Europe, Engels et al. (1981) and Gregersen et al. (1985) proposed that BHV-1 isolates could be assigned to corresponding virus "types" (IBR-like or IPV-like) by restriction endonuclease analysis. These findings could not be confirmed in the case of North American (Misra et al., 1983; Seal et al., 1985) or Australian (Brake and Studdert, 1985) strains. No strict correlation could be detected between the different restriction endonuclease patterns and the clinical origin of the investigated 33 Hungarian BHV-1 isolates either. Metzler et al. (1985) later proposed that isolates originating from cases of respiratory tract disease or abortion displayed either a BHV-1.1 or a BHV-1.2a endonuclease restriction pattern, whereas strains related to genital disease showed BHV-1.2a or 1.2b characteristics. In accordance with these findings, 23 Hungarian respiratory isolates belonged to group 1 or 2a and the two Hungarian IPV isolates (HB124 and HB191) could be assigned to subgroup 2a, but four of the respiratory isolates and a further one isolated from the generalized systemic form of BHV-1 infection were classified as subgroup 2b virus, showing that this virus type is also capable of causing severe symptoms in cattle (Table 2). While all these indicate that restriction enzyme analysis as a tool for the indication of virus organotropism has failed so far, the possibility cannot be ruled out that certain virus types or strains show higher affinity to specific organs. Our previous – unpublished – results showed that following simultaneous infection of the nasal and the vaginal mucosa of 8-week-old calves with BHV-1 strains of different origin (K22–IPV or HB144–IBR), the virus could be recovered with a higher success rate from the vagina if the calf had been infected with K22, and from the nasal mucosa in case of HB144 infection. Similar observations have been described by Whetstone and Miller (1989) after a reinfection experiment with different virus types (strains K22 and Cooper) following dexamethasone provocation of the already latent viruses.

A new virus species, BHV-5, has recently been established for bovine encephalitis herpesvirus strains with a unique restriction cleavage pattern (formerly designated as BHV-1.3 by Engels). Although this virus type has been isolated exclusively from bovine

encephalitis cases so far, BHV-1 has also been isolated from this clinical form along with the classical forms of IBR or IPV, like HB183 in the present study or the German isolate V181 (Meyer and Huebert, 1987). The clinical appearence and many other characteristics of BHV-1 and BHV-5 may overlap, but their polypeptide patterns and restriction cleavage patterns show consistent differences. Group 1 and group 2 viruses share 24 common *Eco*RI, *Hin*dIII, or *Bst*EII restriction cleavage sites with only two cleavage sites being present in one of the two groups only (additionally there are two further subgroup-specific cleavage sites). On the other hand, only nine out of the 24 common cleavage sites are present in group 3 viruses, and 12 other cleavage sites are unique for "group 3" or BHV-5 showing a much more distant relation to groups 1 and 2 than that between. Never the less, the existence of common cleavage sites shows that BHV-5 stands the closest to BHV-1 as a relative in the Herpesviridae family.

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SEROLOGICAL SURVEY FOR TYPE D RETROVIRUSES AND SIMIAN IMMUNODEFICIENCY VIRUS IN MONKEYS LIVING IN HUNGARY

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Acquired immunodeficiency in non-human primates has been associated with two types of retroviruses: (i) Simian Retrovirus (SRV), a type D virus and (ii) a lentivirus, Simian Immunodeficiency Virus (SIV). Both types represent a serious health threat to animals living in different primate research centres, zoos, medical institutions and in private homes. The sera of 175 monkeys belonging to different species were tested for the presence of antibodies against type D retroviruses and SIV. The Mason-Pfizer monkey virus, the prototype of D retroviruses, and Simian Immunodeficiency Virus, designated SIV_{mac251}, were used. None of the sera tested proved to be positive for the presence of type D retroviruses and only one serum of an African green monkey was positive for the presence of antibodies to SIV.

Key words: Type D retrovirus, simian immunodeficiency virus, monkey, serological survey, Hungary

Simian acquired immunodeficiency syndrome (SAIDS) is an immunosuppressive disease of non-human primates. It is characterized by lymphadenopathy, opportunistic infections and defects in humoral and cell-mediated immunity. In the background of the pathological changes type D retroviruses or SIV may be present (Desrosiers, 1988; Gardner et al., 1985). Mason-Pfizer monkey virus (MPMV), the prototype of D retroviruses was isolated in 1970 from a tumor-bearing rhesus monkey (Chopra and Mason, 1970). It was clarified later that there is no connection between this virus and tumor induction. Further two members of this virus group were isolated in the middle of the 'eighties: SRV-1 in 1984 (Daniel et al., 1984) and SRV-2 in 1985 (Marx

et al., 1985). All these three serotypes were found in macaques. In serological tests they cross-react to some extent, providing the possibility to investigate the presence of antibodies against all the three serotypes in common test.

Simian lentiviruses are related to Human Immunodeficiency Virus (HIV). Different isolations from three species of non-human primates are known at present: SIV_{mac} from rhesus monkey with immuno-deficiency syndrome and lymphoma (Daniel et al., 1985), SIV_{AGM} from asymptomatic African green monkey (Ohta el al., 1988) and SIV_{SMM} from sooty mangabey (Lowenstine et al., 1986). Although human and non-human primate lentiviruses have been known only since the beginning of the 'eighties, at present they belong to the most thoroughly explored viruses (Wain-Hobson et al., 1985; Kestler et al., 1988).

The appearance of these viruses or their latent presence in research centres and medical institutions not only constitute a serious health threat but also offer a possible model for AIDS research.

The aim of our study was to obtain data on whether monkeys in Hungarian zoos, medical institutions or private homes are free of or infected with, SRV and SIV.

Materials and methods

Cells. Human lymphoblastoid cell line Raji (Pulvertaft, 1964) and MPMV-producing Raji cells (Raji/MPMV) were kindly provided by V. Wunderlich (Berlin) (Daniel et al., 1984; Fleischhacker et al., 1987). MT4 human T cell line was kindly provided by D. Nosik (Moscow) with the permission of N. Yamamoto (Tokyo). SIV_{mac251} was kindly provided by R. Desrosiers (Soughborough) in HUT78 human T cells. The cells were cultured in RPMI-1640 medium supplemented with 10% fetal calf serum, 100 IU/ml penicillin and 100 μg/ml streptomycin. The cells were subcultured twice weekly keeping the cell number between 0.5–1.5 x 10^6/ml.

Serum samples. Sera were collected from animals older than one year. The samples were kept at –20 °C until used.

Virus. SIV_{mac251} was produced by infecting MT4 cells (MT4 /SIV_{mac251}) with the original supernatant of virus-producing HUT78 cells. Virus production was controlled by measuring reverse transcriptase (RT) activity, by indirect immunofluorescence (IF) with an HIV-positive human serum cross-reacting with SIV antigens, or with a SIV-positive monkey serum.

RT assay. The method used was described by us earlier (Somogyi et al., 1990). Briefly, poly(rA): oligo$(dT)_{12\text{-}18}$ as template primer was immobilized on Whatman DE 81 filter paper squares. ^3H TTP and virus–RT buffer mixture were dropped on the squares. They were incubated for 2 h, dried and cpm was measured in a scintillation cocktail using a Packard 1600 CA liquid scintillation analyser.

Immunofluorescence assay. Indirect IF assay for antibodies against D retroviruses and SIV was carried out as described previously (Gyuris et al., 1990). Briefly, cell smears (Raji, Raji/MPMV and MT4, MT4/SIV respectively) were fixed with cold acetone. The cells were then incubated with PBS-diluted monkey serum (dilutions 1:10 and 1:100). SRV-positive control serum from a *Macaca fascicularis* was provided by I. Klots and SIV-positive serum was provided by S. Voevodin (Sukhumi). After washing, the cells were incubated with FITC-conjugated anti-human IgG. The results were evaluated using an Opton fluorescent microscope.

Western blot analysis. For Western blotting, SIV was concentrated from clarified culture fluid of SIV-infected MT4 cells by ultra-centrifugation (Beckman L7-55 ultracentrifuge, SW40 rotor, 30,000 rpm at 4 °C for 2 h). The virus pellet was mixed with sample buffer, boiled, then electrophoretized on polyacrylamide gradient gel (5–10%) by the method of Laemmli (1970). The polypeptides were blotted on nitrocellulose sheets. Strips were cut from the sheets. Sera to be tested were diluted and incubated with the strips. After washing and incubation with horseradish peroxidase conjugated goat anti-human IgG the reaction was visualized by addition of diaminobenzidine.

Results and discussion

The animals included in the survey are shown in Table 1. A total of 175 sera were tested for antibodies to D retroviruses and SIV. Since indirect IF assay was used as the basic method of testing monkey sera, first of all the virus production of Raji/MPMV and MT4/SIV_{mac251} cells had to be checked. This was done by RT assay. Table 2 clearly shows that both of these cell types are good virus producers, especially MT4/SIV_{mac251}.

Table 1

Monkeys included in the survey

Species animals	Number of
Cercopithecus aethiops (African green monkey)	97
Macaca mulatta (Rhesus monkey)	34
Macaca fascicularis	12
Pan troglodytes (Chimpanzee)	11
Pongo pygmeus (Orang-utan)	1
Papio hamadrias (Baboon)	1
Unknown	19
Total	175

None of the sera proved to be positive in repeated IF test (all sera were tested two times in different series of test slides) for the presence of antibodies against Simian Retrovirus. The faint, uncertain reaction observed for five sera (three macaques, two African green monkeys) proved negative on repeated testing using different dilutions.

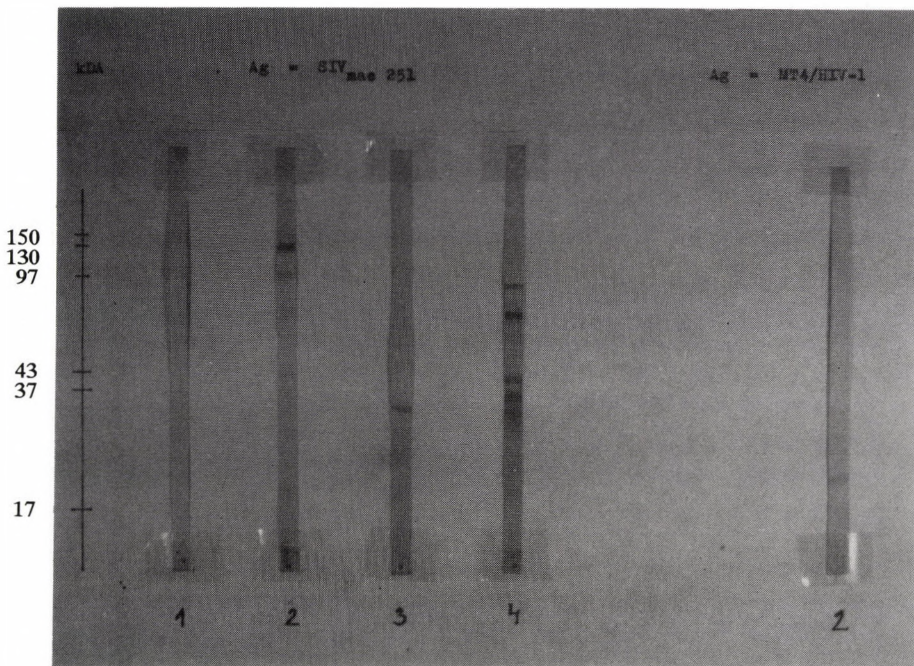

Fig. 1. On the left side of the figure four strips are shown where SIV_{mac251} was used as antigen and incubated with the following sera: (1) negative monkey serum; (2) serum of African green monkey giving positive IF reaction; (3) known positive human serum for HIV-1; and (4) known positive human serum for HIV-2. Serum no. 2 gave a strong positive reaction. HIV-1 and HIV-2 positive human sera (no. 3 and 4) reacted on these strips as well. (SIV and HIV-2 viruses are more closely related than are SIV and HIV-1.) The single strip on the right side shows a reaction between MT4/HIV-1 (antigen) and serum no. 2

Table 2

RT activity measured on the 7th day of culture

Cell	cpm/ml
MT4	313
MT4/SIV_{mac251}	53,993
Raji	275
Raji/MPMV	15,212

Three sera (all from macaques) gave doubtful reaction in the first test for SIV antibodies, and one serum (from an African green monkey) yielded a clear positive reaction. In order to verify these results, in addition to repeating the IF assay we performed Western blotting. The African green monkey serum giving a positive IF proved to be positive in Western blot as well (Fig. 1).

The experiments presented in this paper were performed to get information about the possible presence of non-human primate retroviruses in monkeys living in Hungary. All the animals included in this survey were healthy with no sign of immunodeficiency or opportunistic infections. All the sera investigated were negative for D retroviruses and only one out of the 175 samples was verified as positive for SIV. These results suggest that at present the danger of an SRV–SIV infection of monkeys in Hungary is relatively insignificant. When purchasing new animals for different purposes, however, they should be tested so that the transmission of these viruses could be avoided.

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DETECTION OF BOVINE HERPESVIRUS 1 WITH VARIOUS TYPES OF DNA PROBES

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The method of dot-blot hybridization on nitrocellulose filters by various types of DNA probes (ds recombinant plasmids, ss recombinant M13 phages and a 42bp synthetic oligonucleotide) was used for BHV-1 detection. The highest sensitivity was achieved with the ^{32}P-pUR1 probe (1.8 kb random *Eco*RI-*Hin*dIII fragment inserted into pUC9) which detected the BHV-1 genome in 5 x 10^3 infected MDBK cells. Using the pUR1 probe, no cross hybridization was observed with other herpesviruses: BHV-2, 3, 4, and Aujeszky's disease virus. The ^{32}P-pUR1 probe detected BHV-1 in nasal swabs of calves as early as on day 1 after experimental infection. The maximum intensity of BHV-1 detection occurred on day 1-3. The ^{32}P-pUR1 probe also detected BHV-1 in field samples of nasal swabs from cows and calves.

Key words: Bovine herpesvirus 1, IBR, dot-blot hybridization, DNA probe

Bovine herpesvirus 1 (BHV-1) was isolated in the U. S. A. in the 1950's. It is the aetiological agent of infectious bovine rhinotracheitis (IBR; McKercher et al., 1975) and infectious pustular vulvovaginitis (IPV; Kendrick et al., 1958).

The usual laboratory diagnosis of BHV-1 is based on virus isolation, but various other assays, e.g. specific antibody detection, ELISA, or detection of specific antigens (Van Donkersgoed and Babiuk, 1991) are also used. In spite of the efforts to improve the laboratory diagnosis of IBR the need for a simple, rapid and specific method of

BHV-1 detection still persists. The diagnosis of latent infections presents an especially difficult problem.

The advances made in recombinant DNA techniques in the early 1980's enabled the detection of microorganisms by nucleic acid hybridization methods demonstrating the presence of the microorganism's genome in samples by the use of specific radioactively or non-radioactively labelled DNA/RNA probes (Viscidi and Yolken, 1987). This trend has also affected the diagnosis of infectious diseases in veterinary medicine (Paul, 1990).

Several laboratories have verified BHV-1 infection by virus genome detection using various nucleic acid hybridization techniques (Dorman et al., 1985; Dunn et al., 1986; Rock et al., 1986; Andino et al., 1987; Belák et al., 1988; Pacciarini et al., 1988). The results of these experiments suggested that molecular-genetic methods could also be used for innovating the diagnosis of IBR.

In our work, various types of DNA probes [double stranded DNA (dsDNA), single stranded DNA (ssDNA) and synthetic oligomer] were used, in order to select the one most suitable for the detection of BHV-1 genome in cell cultures and in cattle.

Materials and methods

Viruses and cell cultures. MDBK cells were infected with BHV-1 [strain R-6 from the collection of microorganisms of the Institute of Veterinary Medicine (VUVL), Brno, Czechoslovakia] with an MOI of 1 for 24 h. After infection, the cell suspension and also non-infected cells serving as controls were resuspended and transferred into tubes at concentrations of 10^3, 10^4, 10^5, 10^6, $5x10^6$ and 10^7 cells/tube. After filling up with saline to a volume of 0.5 ml, DNA was isolated from the cells by phenol extraction.

To verify the specificity of the pUR1 probe, DNA isolated from various herpesviruses was used: BHV-1 (strain HB/176), BHV-2 (strain TV), malignant catarrhal fever virus – BHV-3 (strain WC 11),

BHV-4 (strain Móvár 33/63), Aujeszky's disease virus (strain Benatky 86), all from the collection of VUVL, Brno.

Experimental infection of calves. Five clinically healthy 6- to 7-month-old calves with no BHV-1 antibodies were infected intranasally with a BHV-1 suspension of a total titre of $10^{8.5}$ $TCID_{50}ml^{-1}$. Nasal swabs were collected from day 0 to day 7 after infection. The body temperature was measured and the clinical signs of IBR observed.

Field samples. Nasal swabs were collected from calves and cows on various farms with BHV-1 infection (serological detection of specific antibody).

Collection of nasal swabs. Nasal swabs were collected by profound rotatory movements with a cotton tampoon. The tampoon was put into 1.5 ml saline with protective antibiotics and left there overnight at 4 °C. The solution was then pressed out of the cotton; 0.5 ml of it was taken and the DNA was isolated by phenol extraction. The other part of the samples was used for detecting BHV-1 by virus isolation.

DNA isolation and denaturation. Fifty μl of 10 % SDS and 5 μl proteinase K (20 mg/ml) were added to a 500 μl sample and incubated for 1-1.5 h at 37 °C. The aqueous solution was then extracted twice equal volumes of phenol and re-extracted with phenol–chloroform (1:1); DNA was precipitated from the aqueous phase with 2 vol. ethanol in the presence of 0.25 M CH_3COONa, pH 5.2. DNA was dissolved in 50–100 μl TE buffer, and a 5-μl volume of the solution was used for hybridization. Prior to blotting onto nitrocellulose filter (S & S, BA/85), DNA was denatured either by heat shock or by the alkaline method (Maniatis et al., 1982).

DNA probes. The following probes were used for hybridization: (a) pUR1 recombinant plasmid contains a 1.8 kb *Eco*RI-*Hin*dIII random fragment of BHV-1 DNA inserted into pUC9. (pUR1 was kindly provided by the Laboratory of Genetic Engineering of the Moscow Veterinary Academy.); (b) pUR2: a 4.8 kb *Sal*I-*Pst*I fragment of BHV-1 DNA containing 2/3 of one of the virus immediate early (IE) gene inserted into pAT153; (c) Single stranded form of the phage M13mp18 inserted with a 443 bp (probe pMR1) or 273 bp (probe pMR2) frag-

ment of the 3'region of an IE gene region; (d) A 42 bp synthetic DNA oligomer (5'-GGCGTCATTTACAAGGAGAACATCGCGCCGTA-CACGTTCAAG-3') representing part of the gene of the immunologically important glycoprotein gI (Whitbeck et al., 1988).

Double stranded DNA was labelled by nick translation (kit by Amersham) using (α-^{32}P)-dCTP (Izinta, Hungary); single stranded DNA was labelled by multiprime labelling (kit by Amersham); and for the synthetic oligomer, 5'-end labelling with (gamma-^{32}P)-ATP (Izinta, Hungary) was used. Prior to hybridization, dsDNA probes were denatured by the alkaline method (Maniatis et al., 1982).

Dot-blot hybridization. Five μl samples of denatured DNA were blotted onto nitrocellulose (NC) membranes. The filter was dried and heated to 80 °C in a vacuum dryer for 2 h. Pre-hybridization was done in 6 x SSC, 0.5 % SDS, 5 x Denhart solution, containing 100 μg/ml denatured salmon sperm DNA at 68 °C for 2–3 h.

For hybridization (68 °C/18 h), the same solution was used, containing a denatured ^{32}P-DNA probe (100 ng) instead of heterologous DNA. The pre-hybridization and hybridization temperature was decreased to 58 °C for hybridization with the synthetic 42 bp DNA oligomer. After hybridization, the NC membranes were washed for 15 min at 20 °C in a solution of 1 x SSC, 0.5 % SDS; for 15 min at 20 °C in 1 x SSC, 0.1 % SDS; and for 3 x 20 min at 68 °C (for 58 °C for the 42 bp oligomer) in 0.1 x SSC, 0.5 % SDS. After drying, radioactive signals were detected on X-ray film by autoradiography for 1–3 days.

Virus isolation from nasal swabs. Isolation of the virus was carried out on primary fetal calf kidney cells by observation of the cytopathic effect.

Results

The presence of BHV-1 in the infected cell lines could be proved by dot-blot hybridization on nitrocellose filters with all ^{32}P-DNA probes. No positive signals could be seen in non-infected MDBK cells. However, at a concentration of 10^7 cells/sample a slight nonspecific

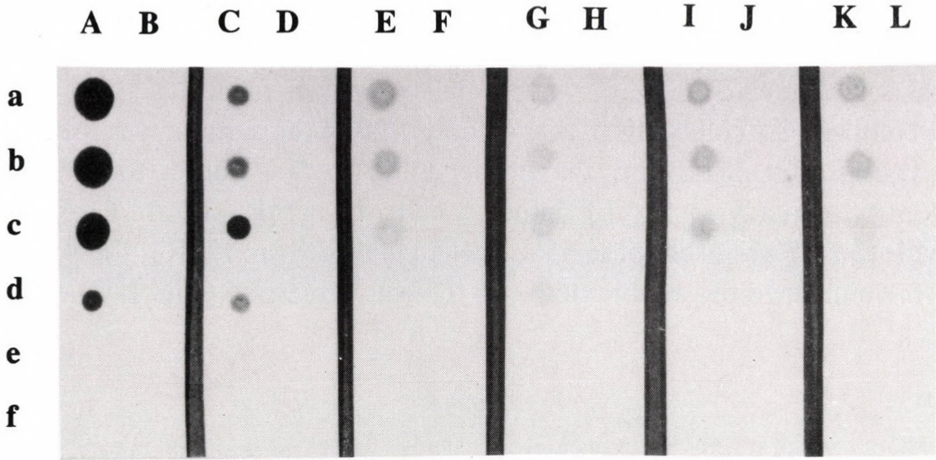

Fig. 1. Detection of BHV-1 in experimentally infected MDBK cells by dot-blot hybridization with various ^{32}P-DNA probes. Probes: A, B - pUR1; C, D - 1.8 kb *Eco*RI-*Hin*dIII fragment of BHV-1 DNA; E, F - pUR2; G, H - pMR1; I, J - pMR2; K, L - 42 bp DNA oligonucleotide. Cells: A, C, E, G, I, K - infected cells; B, D, F, H, J, L - control MDBK cells; a - 10^7, b - 5×10^6, c - 10^6, d - 10^5, e - 10^4, f - 10^3 cells

signal occurred. This nonspecific reaction was most pronounced in hybridization with the synthetic oligomer (Fig. 1, L). The highest sensitivity was achieved with the pUR1 recombinant plasmid. This probe reliably detected the virus in samples prepared from 10^5 BHV-1 infected MDBK cells (Fig. 1, A). As only 1/20 of the sample was used for blotting onto NC filter, the probe could in fact detect the BHV-1 genome already in 5 x 10^3 infected cells. In hybridization of samples with the 1.8 kb insert of BHV-1 DNA cleaved from pUR1, the results were similar to those obtained with the whole plasmid (Fig. 1, C).

Table 1

Comparison of various methods for the diagnosis of BHV-1 infection in experimentally infected cattle

Method	Day after infection							
	0	1	2	3	4	5	6	7
VI	-	+	+	+	+	+	+	+
DBH	-	+	+	+	+	+	+	-
T	-	-	+	+	+	+	+	+
CS	-	-	-	+	+	+	+	+

Results of analysis of 5 animals; VI: virus isolation; DBH: dot-blot hybridization; T: body temperature, + above 39.5 °C; CS: clinical signs (general apathy, respiratory symptoms)

Using the pUR1 probe, no cross-hybridization reactions could be observed with herpesviruses BHV-2, 3, 4, and Aujeszky's disease virus (results not shown).

The usefulness of the ^{32}P-pUR1 probe was further verified by BHV-1 detection in nasal swabs from experimentally infected calves. Dot-blot hybridization proved the presence of BHV-1 in nasal swabs of calves as early as on day 1 after infection. Maximum occurrence of the infectious agent varied in the animals (Fig. 2), i.e. on day 1 (calf no. 4),

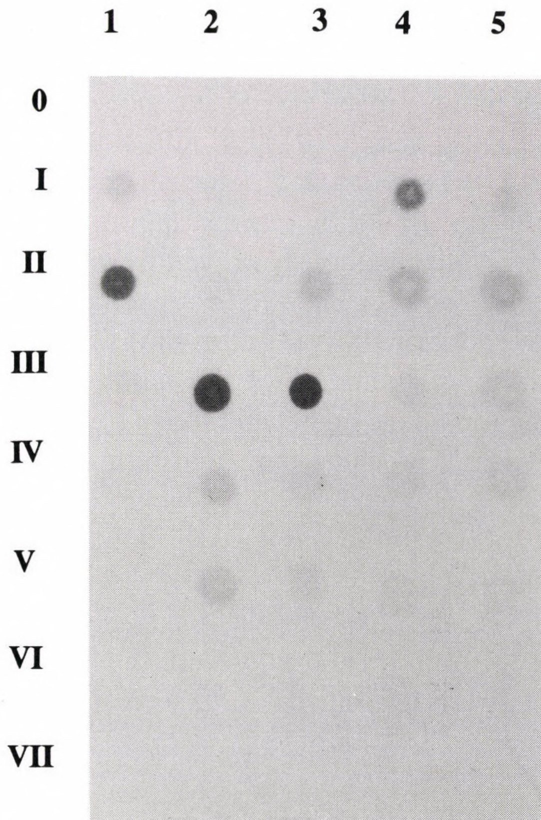

Fig. 2. Detection of BHV-1 in nasal swabs of experimentally infected calves by dot-blot hybridization with ^{32}P-pUR1 probe. 1–5: number of infected calf, 0–VII: day 0–7 after infection

day 2 (calves no. 1 and 5), and day 3 (calves no. 2 and 3), respectively. BHV-1 could not be detected in any of the animals after day 6 or 7.

Virus isolation proved the occurrence of the infectious agent in nasal swabs as early as on day 1 after infection. The largest amounts of virus were isolated on day 2–5; later on the quantity of BHV-1 decreased. In contrast to dot-blot hybridization, virus occurrence in nasal swabs was more unambiguously proved by virus isolation even as late as on day 6 or 7 (Table 1). Elevated body temperature was observed on day 1, achieving maximum values on days 2–4. Clinical signs of IBR (general apathy, inflammation of the nasal mucosa, nasal secretions later assuming a mucous and even bloody nature, red noses) could be observed on day 3, reaching their maximum on day 6 after infection (Table 1).

The ^{32}P-pUR1 probe was used for detecting BHV-1 in field samples, i.e. in the nasal swabs of cows and calves suspected of being infected by BHV-1. Positive hybridization signals were obtained in some animals (Fig. 3, A). In hybridization with the ^{32}P-1.8 kb cleaved BHV-1 DNA insert of pUR1, positive results were not confirmed in all cases (Fig. 3, B).

Discussion

One of the most important components of nucleic acid hybridization is the specific probe. In the case of BHV-1 detection, the considerable length of the viral genome (138 kb) offers a choice of several potentially specific probes. Their diagnostic value must be verified by hybridization experiments that would confirm specificity and sensitivity. For BHV-1 DNA detection probes, most authors used recombinant bacterial plasmids containing randomly cloned fragments of the BHV-1 DNA. For example, in dot-blot hybridization, Dorman et al. (1985) used the pCB2 plasmid prepared by cloning of the 9.6 kb *Hin*dIII fragment I of the BHV-1 genome into pBR325; while for *in situ* hybridization, Dunn et al. (1986) used the pCB31 plasmid containing the *Hin*dIII fragment C of BHV-1. For direct filter hybridiza-

A

B

Fig. 3. Detection of BHV-1 in field samples of nasal swabs from calves and cows using ^{32}P-pUR1 (A) or its ^{32}P labelled 1.8 kb *Eco*RI-*Hin*dIII fragment (B)

tion (which is a modification of dot-blot hybridization), Belák et al. (1988) used the pBH5 plasmid with a 5.4 kb random *Pst*I insert and the pBH132 plasmid with two *Pst*I inserts, 3.08 and 1.3 kb, respectively, inserted into pKH47. Italian authors (Pacciarini et al., 1988) could detect BHV-1 virus by dot-blot hybridization in the semen of bulls using the pHV2 plasmid which contained a 12.5 kb *Hin*dIII fragment inserted into pUC8. Three different recombinant plasmids – pHBB3, pHBB4 and pHBB5 with 8.9, 15.5 and 17.8 kb *Bam*HI inserts in pAT – were also used by other authors for BHV-1 detection (Andino et al., 1987).

Various types of DNA probes were used for BHV-1 detection in our experiments: two double stranded viral DNA fragments of different length in plasmids; two short fragments of the BHV-1 genome present in the single stranded form of the M13 phage; and a synthetic 42 bp oligonucleotide representing part of the gene encoding the immunologically important gI protein. The highest BHV-1 detection sensitivity was achieved with the pUR1 probe constructed by the insertion of a random 1.8 kb *Hin*dIII-*Eco*RI fragment of BHV-1 into pUC9. As this probe did not react with other herpesviruses, it was used in further experiments. In spite of that, DNA probes using the M13 phage may have a better perspective. The occurrence of the M13 phage in clinical samples (e.g. nasal swabs of calves) is less probable than that of bacterial plasmids. This decreases the risk of a false-positive reaction. In order to increase the sensitivity of a DNA probe based on the ss form of the M13 phage, it will be necessary to choose a more suitable BHV-1 DNA insert.

From our results it follows that by means of dot-blot hybridization even very early phases of infection can be detected. Application of ^{32}P-pUR1 enabled us to prove BHV-1 infection in experimentally infected calves as early as on day 1 after infection, in the same way as by virus isolation in cell cultures. In the animals, clinical signs were seen as late as on day 3–4. Maximum infection could be observed with ^{32}P-pUR1 on day 2–3; virus isolation revealed this maximum on day 2–5. A decreased occurrence of BHV-1 in nasal swabs was observed on day 6–7 by both methods.

It should be pointed out that the virus isolation technique could prove the presence of virus also in the later phase (day 6–7 in our experiments) when dot-blot hybridization did not yield unambiguous results. This relation was observed also by Belák et al. (1988).

Our experiments demonstrated the possibility of using the pUR1 probe in the laboratory detection of BHV-1 in field samples of nasal swabs from calves and cows with naturally occurring IBR. However, taking into account the possibility of false-positive reactions, it would be suitable to verify the positive samples either by hybridization with the cleaved virus insert of the plasmid or by a negative result of hybridization with the original vector without an insert.

The latest trend in the detection of microorganisms is the use of the PCR method based on *in vitro* amplification of a chosen genome fragment (Saiki et al., 1985). Since the PCR method is suitable for the detection of latent infections (Maes et al., 1990), this method was also developed in our laboratory. The amplification of a 468 bp DNA fragment of BHV-1 was successful (Vilček, 1992).

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CONTENTS

Animal nutrition

Complex study of the physiological role of aluminium. I. Review of the literature. *Bokori, J.* and *Fekete, S.* 193
Complex study of the physiological role of aluminium. II. Aluminium tolerance tests in broiler chickens. *Bokori, J., Fekete, S., Kádár, I., Vetési, F.* and *Albert, M.* 235
The role of feed protein quality in reducing environmental pollution by lowering nitrogen excretion I. Survey of principles in protein evaluation for monogastrics: A review. *Hegedűs, M.* 265
The role of feed protein quality in reducing environmental pollution by lowering nitrogen excretion II. Choice of an assay for monitoring protein quality and predicting protein utilization. *Hegedűs, M.* 283

Animal reproduction

Differences in biochemical profile from active and inactive ovarian tissue in relation to oestrous cycle phases. *Sureshkumar, P. K.* and *Janakiraman, K.* 301

Bacteriology

Comparative pathogenicity study of *Leptospira interrogans* serovar *pomona* strains. *Nagy, Gy.* 315

Epizootiology

Hungary remains free of scrapie and bovine spongiform encephalopathy (BSE). *Kluge, J. P.* and *Glávits, R.* 325

Genetics

Influence of parental origin, litter size and sex on the frequency of splayleg in piglets: A case report. *Tomko, M.* 329

Parasitology

Sphaerospora siluri n. sp. (*Myxosporea: Sphaerosporidae*) in the kidney of the sheatfish (*Silurus glanis*). *Molnár, K.* 341
Effect of decreased oxygen content on eels (*Anguilla anguilla*) infected by *Anguillicola crassus* (Nematoda: Dracuncoloidea). *Molnár, K.* 349
Efficacy of Butalex in field cases of bovine theileriosis — Short communication. *Mishra, A. K., Sharma, N. N.* and *Viswanathan, C. B.* 361

MAGYAR
TUDOMÁNYOS AKADÉMIA
KÖNYVTÁRA

Pathophysiology

Concurrent oral administration of monensin and selenium to broiler chickens: Effects on concentration of different elements in the liver. *Khan, M. Z., Szarek, J.* and *Markiewicz, K.* 365

Physiology

Thyroid hormone deiodination in the brain of young chickens acutely adapts to changes in thyroid status. *Rudas, P., Bartha, T.* and *Frenyó, V. L.* 381

Thyroxine and triiodothyronine uptake by the brain of chickens. *Rudas, P.* and *Bartha, T.* 395

Toxicology

Biochemical examination of muscle samples from pheasant embryos affected by Wofatox 50 EC (50% parathion-methyl). *Várnagy, L.* 409

Studies on the toxic and immunosuppressive effects of cadmium on the common carp. *Sövényi, J.* and *Szakolczai, J.* 415

Immunology

T-cell deficiency in Suffolk lambs. *Zomborszky, Z., Horn, É., Tuboly, S., Megyeri, Z., Tilly, P. and Szabó, Cs.* 427

Book reviews 437

Acta Veterinaria Hungarica 41 (3–4), pp. 193–233 (1993)

COMPLEX STUDY OF THE PHYSIOLOGICAL ROLE OF ALUMINIUM
I. REVIEW OF THE LITERATURE

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(Received April 22, 1993)

1. Aluminium and its physiological role

1.1. The occurrence of aluminium

Aluminium (Al) is an ubiquitous metal which constitutes about 8% of the earth's crust as its third most abundant element (Hem, 1985). In contrast, in biological organisms it can be found only in traces.

Most compounds of aluminium are insoluble. In sea-water Al can be detected at a concentration of 1 μg/l, while in freshwaters the concentration of dissolved Al is higher than that (Hem, 1986). Thus e.g. the water of some Northern European lakes was found to contain higher levels of Al as a result of the acid rains (Miller et al., 1984). Higher levels of Al usually occur in freshwaters and drinking-water treated with aluminium sulphate against turbidity (Miller et al., 1984).

According to the studies of Pang et al. (1987), in the waters the UV rays may liberate the organically bound Al which, thus, may reach levels toxic to aquatic organisms including fish.

In small quantities Al occurs also in plants. In certain subtropical plants it may accumulate in especially high concentrations (Chenery, 1984). Thus e.g. in the ash of *Lycopidiaceae* spp. an Al concentration as high as 70% was measured (Hutchinson and Wollack, 1943). Alga meals also contain high levels (3.9–5.3%) of Al (Lipstein and Hurwitz, 1981). A substantial increase in Al content was observed in the root system and fruit of lemon trees sprayed with an Al-containing fluid.

The aluminium tolerance of plants depends on several factors including the species and variety of the plant, the chemical forms of Al, the status

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of the detoxifying mechanism, the Ca and P supply of the organism, the structure and condition of the cell membranes (Merbach, 1986).

Plants that can stand acidic soils better (e.g. rice, yellow lupine) are characterized by higher tolerance to the toxic effects of aluminium (Horst and Göppel, 1986). Taylor and Foy (1985) found that the Mg and Mn uptake of wheat was decreased by Al treatment.

If the dissolved Al content of the soil exceeds 3 μM, it will exert an inhibitory effect on the root growth of plants (Wright and Wright, 1987).

According to Soós et al. (1985), the Al concentration of certain Al-accumulating vegetables (e.g. celery, parsley) changes as a function of environmental pollution with Al. Thus, by measuring the Al content of these vegetables the Al pollution of a given region or area can be inferred.

By *in vitro* experiments, Godbold et al. (1988) demonstrated that the root growth of young plants placed into a nutrient medium containing 500 μM Al per litre was markedly inhibited.

Aluminium enters the animal organism with the feed via the digestive tract or with the air inhaled into the lungs. Free-living animals normally take up 2–5 mg Al with the feed daily (Alfrey, 1983). Depending on the content and quality of Al in drinking-water, varying amounts of Al can get into the animal organism with the drinking-water as well. Aluminium is eliminated from the organism mainly via the faeces but a small amount is secreted into the milk, too. Sedman et al. (1985) found that the average Al content of cow's milk was 266 μg/l. According to a survey involving nine countries, milk substitutes contained much higher levels of Al (77.1–159.0 μg/100 g milk powder).

Feldspars like kaolin and bentonite, which contain Al compounds usually occurring in complex form in clayey soils, can be used for several purposes in animal nutrition. They are used for pelleting as binding material, for the prevention of clumping and dust formation in industrial mixed feeds, and owing to their favourable effect exerted on feed utilization and on the mucous membrane lining the gastrointestinal tract.

1.2. The essentiality of aluminium

Earlier Al was considered a completely indifferent substance and it has purposely not been added to the diets of animals to this day. However,

as a result of the altered environmental conditions now it is getting into the living organisms in ever increasing amounts. Due to this fact, it commands an increasing interest and several of its physiological effects have been described. Research efforts aimed at exploring the biological role of Al were launched in the 1940's, and between 1977 and 1984 the number of publications dealing with the physiological effects of that metal exceeded 300 (Mertz, 1986).

Studies on the essentiality of aluminium are justified, besides the above reasons, also by the high prevalence of Al on planets of the solar system and its place occupied in the periodic system of elements. Numerous studies have demonstrated that Al has many important functions in the vital processes of both plants and animals. As in the case of plants primarily the negative effects exerted by Al on cell division and on the uptake as well as metabolism of other elements such as Mn, Mg and P have been described (Horst et al., 1983; Taylor and Foy, 1985), Al has been reported to have several favourable physiological effects in animals. Its presence has been reported to further the reaction between cytochrome c and succinyl dehydrogenase (Horecker et al., 1939). Aluminium is a cofactor necessary for the activation of guanine-nucleotide binding which is important in protein metabolism (Sternweis and Gilman, 1982). It has been shown to play an important role in the development of potent immune response and in endogenous triglyceride metabolism (Sugawara et al., 1987). In the abovementioned physiological effects Al is involved primarily as a qualitative rather than a quantitative factor (Pais, 1989).

1.3. The metabolism of aluminium

As the environment is abundant in Al, this element can continuously get into the animal organism both with the feed and drinking-water and via the lungs and skin. Despite this fact, usually little Al is absorbed into, and retained in the organism. In view of the fact that Al has no isotopes, its metabolism has remained unclear in several respects. However, by indirect methods, primarily by experiments on rats, mice and rabbits, a rather large body of knowledge has been accumulated on the absorption, storage and excretion of Al.

Aluminium may enter the organism basically in two ways, via the digestive tract and the respiratory tract, as a feed constituent or environmental pollutant, and parenterally. The latter can take place in several different ways and is an important route of uptake (3–5 mg/day) mainly in humans (Gorsky and Dietz, 1979; Gorsky et al., 1979*a,b*).

The chemical form of Al and the amount of Al taken up play an important role in the absorption of otherwise poorly soluble Al compounds taken up with the feed and drinking-water (and possibly with drugs). Thus, the phosphorus and fluorine compounds of Al show poor absorption (Caster and Wang, 1984), while its hydroxides and chlorides absorb more efficiently. Similarly, Al absorbs better from its complex formed with F. Only minimal increase in the Al concentration of the blood plasma was observed during absorption (Kaehny et al., 1977). The uptake of higher doses of Al was found to result in enhanced absorption. Thus, e.g. in humans the Al content of the urine increased after the uptake of 3–5 g Al per day. Recker et al. (1977) found that after the uptake of 3–8 g Al the amount of Al excreted in the urine by the adult organism increased from 86 μg to 495 μg daily. Similar observations have been reported also by other authors (Greger and Baier, 1983; Spencer et al., 1977), along with a substantial Al retention in the organism. The average amount of Al in the human organism was found to be 60 mg (International Commission on Radiological Protection, 1975). The average daily Al intake of humans with foods has been found to be 2–10 mg. When using aluminium utensils and tableware or taking Al-containing drugs, the Al intake may reach 40 mg (Greger and Baier, 1983). Much aluminium may get into the organism with tomato sauce cooked or stored in aluminium pots or cans. The Al content of Pepsi Cola in cans may reach 30–40 μM (Cho and Joshi, 1987).

Another important factor increasing the absorption of Al is the pH value prevailing in the intestinal tract. It has been unambiguously demonstrated that the absorption of Al is higher from an acidic medium. Thus, the main site of absorption is the stomach but first of all the anterior part of the small intestine (Kaehny et al., 1977). The role of pH is supported by the observation that simultaneously administered citric acid increases the amount of absorbed Al both in man and in animals (Slanina et al., 1984).

The role played in the absorption of Al by the interaction of different elements is important but insufficiently known. Wenk and Stemmer (1983) found that the low Zn and Si content of the feed enhanced the absorption

of Al and, indirectly, the Al content of the brain. In a feeding trial with rats, Brown and Schwartz (1992) studied the effect of Fe on the absorption and detection of Al. They found that the Al content of certain organs (serum, spleen, liver) increased in animals kept on an iron-deficient diet. The increase in Al content was especially pronounced if the diet contained 3.6% sodium citrate in addition to 2% Al ($AlCl_3$). At the same time, the studies of Greger and Baier (1983) failed to unambiguously demonstrate a negative effect of Al on the utilization of several elements (Ca, P, Mg, Zn, Fe and Cu).

The results of experiments on chickens and rats indicated that both 1,25-dihydroxi-cholecalciferol (Long et al., 1980) and PTH increased the Al concentration of the tissues in experimental animals (Mayor et al., 1980). Lewis-Finch et al. (1986) could not corroborate this finding. According to recent studies, only in patients suffering from the terminal stage of renal disease does vitamin D enhance the absorption of Al (Demontis et al., 1989).

Several data are available on how much of the absorbed Al *accumulates* in the different organs. Al is stored mainly in the liver, spleen and bones: in these organs its quantity may reach 300–400 mg per one kg wet mass (Alfrey, 1980; Alfrey et al., 1980). In other organs, e.g. in the brain and heart muscle, much lower Al concentrations (40–50 mg/kg) could be measured. The amount of Al measured in the skeletal muscles was 4–6 mg per one kg dry matter. The Al content of the skin and haircoat showed no correlation with that of the organism, which was presumably due to the fact that these organs are more exposed to environmental contamination (Alfrey, 1980). From this it follows that the Al content of the inner organs is a better indicator of the Al load than that of the entire organism or of the haircoat (Mayor et al., 1980).

Despite the lack of a close correlation between the Al content of the individual organs, the high Al content of bones is usually suggestive of Al contamination of the other organs. However, neither this can be considered a rule of universal validity, as e.g. in uraemic animals the Al content of the bones while in healthy animals that of the liver increased first. All this can be explained by the hyperparathyroid status of diseased animals (Alfrey et al., 1985).

In some experiments the following observations were made on aluminium accumulation.

During prolonged administration of Al-containing antacids to patients being at a terminal stage of liver diseases, substantial accumulation of Al was observed on the surface of bone trabecules (Williams et al., 1986). In contrast, in other experiments the accumulation of Al could not be demonstrated in the bones by histochemical methods after prolonged administration of Al-containing preparations, though the signs of osteomalacia were present (Carmichael et al., 1984; Godsall, 1984). It has also been observed that the administration of large doses of "antacid" preparations to patients suffering from peptic ulcer and osteoporosis increased the Al content of bones (Recker et al., 1977). Klein et al. (1989*b*) administered buffered aluminium citrate into the duodenum at a dose of 100 mg elemental Al/kg over a period of 14 days, and monitored the amount of stored and excreted Al. The Al content of both the serum and the urine substantially increased, while that of the liver increased by only 0.5%. Slanina et al. (1985) observed a substantial increase in the Al content of the brain and bones in rats given an acidic fruit juice prepared in an aluminium pot. This occurred mainly if renal function was insufficient. Similarly, an accumulation of Al was observed in the liver and bones of nephropathic patients administered a solution of phosphate-binding $Al(OH)_3$ and sodium citrate (Sedman et al., 1984). Salusky et al. (1984) reported that orally administered $Al(OH)_3$ significantly increases the Al content of the serum. Recently an increase in the Al content of the bones and the brain has been observed in a nephropathic patient treated with an Al-containing solution supplemented with citrate (Kirschbaum and Schoolwerth, 1989).

In summary, it can be established that in subjects with normal renal function only small amounts of Al are accumulated in the tissues after oral administration. However, substantial Al accumulation will take place if renal function has been impaired.

The absorption of Al and its accumulation within the organism have been studied also after *parenteral Al administration*. In that case, the "intestinal barrier" is short-circuited and, thus, more Al gets into the different organs and accumulates in them also in the case of normal renal function. This is why in nephropathic patients subjected to haemodialysis substantial Al accumulation is observed in different organs, first of all in the bones, liver, spleen, brain and parathyroid gland (Alfrey et al., 1976*a*, 1980).

After prolonged *intravenous administration* of Al-containing fluid to subjects with satisfactory renal function, a substantial amount of Al accumulated in the bones, liver, spleen and blood plasma even if the urinary excretion of Al was enhanced. Klein et al. (1982*b*) observed that 40–60% of intravenously administered Al was retained in the organism even in gastroenteritic patients. One of the factors accounting for this higher Al retention is that Al, 95% of which is bound to plasma proteins (transferrin), is excreted through the renal glomeruli only in small amounts, or not at all (Trapp, 1986). Henry and Norman (1985) observed that Al binds to proteins in a very short time after injection.

Drescher et al. (1986) published the Al content of 10 different organs of deceased patients who had been treated with an Al-containing dialyzing fluid for 1-10 years.

After *intraperitoneal administration*, only little Al is absorbed from the Al-containing fluid, and Al accumulation in the bones is low if renal function is satisfactory (Salusky et al., 1988). In contrast, Chan et al. (1983) found that Al-containing fluid intraperitoneally administered to rats resulted in a substantial Al accumulation in the bones and liver both in uraemic and non-uraemic animals. The results of that experiment indicate that intraperitoneally administered Al may be another possible route leading to the accumulation of Al in the tissues (Ellis et al., 1979).

Few data are available on the accumulation of *subcutaneously administered* Al in the tissues. Yokel (1984) injected different doses of aluminium lactate under the skin of rabbits on days 4–29 post partum. On day 15 the milk contained Al in a relatively high concentration (6 mg/l). Substantial Al accumulation was found in the bones, kidneys and liver. Other authors (Gorsky et al., 1979*a, b*) determined, and found elevated, the amount of Al excreted in the urine after similar Al administration.

These experimental results led researchers to conclude that subcutaneously administered Al results in moderate Al accumulation in the tissues, which manifests itself also in excess Al excretion in the urine. The long-term consequences of such slow Al accumulation are not known yet.

The intramuscular administration of Al may occur in human medicine during vaccination against diphtheria, tetanus and other diseases. The amount of Al in the different vaccines varies between wide limits (34–505 μg; May et al., 1986). Thus, e.g. an infant administered 0.5 ml vaccine intramuscularly takes up approx. 250 μg Al. The degree of tissue deposition

and accumulation and the rate of excretion have not been described in such cases yet.

In summary, it can be stated that orally ingested and parenterally administered Al will accumulate in the tissues, particularly if renal function has been impaired. Aluminium administered by various parenteral routes (subcutaneously, intramuscularly or intraperitoneally) may also accumulate in the tissues, independently of the renal function status.

The excretion of Al has mostly been studied after parenteral aluminium administration. It has been established that the main organ of Al excretion is the kidney. If renal function is normal, the kidney can excrete large amounts of Al, despite the fact that protein-bound Al gets through the glomeruli with rather much difficulty (Henry et al., 1985).

Clashing opinions have been published on the excretion of Al with the bile. Thus, Gorsky et al. (1979*a*) reported substantial biliary excretion of Al after oral ingestion of the element. However, one must keep in mind that in that case the Al ingested with the food but not absorbed was not differentiated from the Al fraction absorbed and excreted in the bile.

Recently it has been reported (Klein et al., 1989*a*) that Al administered to rats at a daily dose of 100 mg/kg body mass is mostly excreted through the kidney, with the urine, and only 2–3% of that amount was excreted in the bile. Similar observations have been made also by other authors. In dogs administered an Al-containing fluid by the intravenous route the amount of Al excreted in the bile was negligible (Kovalchik et al., 1978).

2. The effect of aluminium on different tissues

Researchers have studied the effect exerted by Al on several tissues. On the basis of clinical signs observed during these experiments they tried to reveal the role of Al in the development of the pathological lesions observed.

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2.1. Bones and parathyroid gland

It has been shown that the prolonged administration of Al leads to suppressed bone formation and then gradually results in osteomalacia (Ellis et al., 1979; Goodman, 1984). Aluminium that has accumulated in the calcification zone of bones does not exert a direct pathological effect; rather, it causes an osteopathy through its adverse influence on osteoblasts (Plachot et al., 1984; Lieberherr et al., 1987). According to other authors (Blair et al., 1989; Ott et al., 1987), Al causes bone disorders through its inhibitory effect exerted on the hormone production of the parathyroid gland, or by directly inhibiting the physiological effects of parathyroid hormone (Cann et al., 1979; Klein et al., 1987; Klein et al., 1986). Vitamin D, 1,25-dihydroxi-cholecalciferol may also play an important role in the development of bone abnormities (Klein et al., 1987).

Achenbach et al. (1986) studied the correlation between the effect of Al and changes in bone metabolism in 184 chronic haemodialysis patients. According to their observations, prolonged Al accumulation in the organism inhibited bone mineralization and bone formation, resulting in the painfulness and fragility of bones. In their view, an accurate diagnosis is difficult to establish as the serum concentrations of Ca, P, alkaline phosphatase and parathyroid hormone (PTH) show irregular fluctuations.

In addition, Al may contribute to the development of bone formation disturbances also physically, through inhibiting hydroxyapatite or $CaPO_4$ crystal formation (Meyer and Thomas, 1986). Further studies are needed to get a closer insight into the role played by aluminium in bone formation.

2.2. Liver

The effect of aluminium on liver cells has been studied by several authors. Klein et al. (1987) administered aluminium in dissolved form to piglets intravenously over a period of eight weeks. They found that the Al concentration of both the bile acids and the blood increased. Electron microscopic studies revealed that Al had bound to the lysosomes (Klein et al., 1989). No other ultrastructural change could be detected. The excretion of Al with the bile acids could be prevented if Al was administered at low doses (Klein et al., 1986). When injected intravenously in large doses, Al

caused cholestasis. Bidlack et al. (1987) reported that the intravenously administered aluminium solution enhances the activity of inactive glucuronyl transferase. It is believed that Al bound to microsomal enzymes is capable of damaging the cell membranes, resulting in the enhanced liberation of cellular enzymes. This pathophysiological effect of Al must be studied further.

2.3. Kidney

Only few data are available on the effect of Al on the structure and function of the kidneys. It was found that Al intraperitoneally administered to rats with normal renal function prevents phosphate excretion (Henry and Norman, 1985 ; Klein et al., 1988) in the same way as does an intravenously administered parathyroid hormon analogue. Conflicting views have been published on the effect of Al on vitamin D_3. According to some authors (Henry and Norman, 1985), Al inhibits the activity of vitamin D_3, while others (Sedman et al., 1985, 1987) could not demonstrate such an effect. It is unclear whether this effect is specific, dose dependent, or both.

2.4. Brain

Local Al administration was shown to result in "mental" disorders in monkeys (Kopeloff et al., 1942). However, many questions have remained unanswered as regards the neurotoxic and neurodegenerative effect of Al. Ribak et al. (1979) established that an intracortically administered Al solution not only exerted a local and systemic effect but also reduced the number of synapses involved in the transmission of nervous impulse and utilizing alpha-aminobutyric acids.

According to other authors (Marquis and Black, 1984), Al may inhibit the activity of acetylcholinesterase, thus influencing the Ca level of the tissues and cytoplasm. In rabbits, the administration of Al causes progressive encephalopathy associated with neurofibrillar degeneration. This lesion, however, does not fully correspond to the changes seen in Alzheimer's disease in human medicine. Similar lesions were consistently produced in cats by intracerebrally administered Al (Crapper et al., 1980).

The neurological lesions seen in rabbits and cats were successfully produced by Al administration in cerebrocortical cell cultures *in vitro* (De Boni et al., 1980). After injecting Al into the cisterna magna of rabbits, Wisniewsky et al. (1984) observed the swelling of axons and the thickening of dendrites. Several authors observed an elevated Al content of the brain in rabbits after the subcutaneous administration of Al solution (Uemura, 1984), as well as a concurrent effect of several elements (Zn, Ca, Si) on the Al content of the brain (Carlisle and Curran, 1987; Garruto et al., 1988). The observed pathological lesions resembled those seen in Parkinson's disease and in sclerosis. Garruto et al. (1988) also observed these lesions but could not determine whether they resulted from a direct effect of Al or were due to secondary Ca deficiency. By Al administration during Ca-deficient feeding, Yase (1988) successfully produced the lesions in rabbits; however, he did not check the effect of low Ca without Al administration.

Banks and Kastin (1983) attribute the nervous lesions produced by orally administered Al to the altered permeability of the blood–brain barrier. According to Perl and Good (1987), Al getting into the brain through the olfactory nerve also may account for the neurological lesions. This view is supported by the observation that the administration of Al into the olfactory bulb of rabbits led to the development of Al granulomas both in the olfactory bulb and in the cortex. Another possibility is that Al gets into the brain by first binding to plasma transferrin, and it binds to nerve cells after crossing the endothelial lining which forms the blood–brain barrier (Wills and Savory, 1989). According to the studies of Zumkley et al. (1986), after the administration of antacid preparations rich in Al the Al content of the brain rapidly increased to as high levels as twice the original value (from 0.583 μg to 1.07 μg in 1 g crude tissue). Levels as high as that may already cause aluminium encephalopathy.

In summary, it can be established that the direct administration of Al into the central nervous system may result in severe pathological lesions including neurofibrillar degeneration, neurofilament formation, encephalopathy, and changes in the synthesis and/or activity of neurotransmitter substances. In sensitive species, these lesions could be observed after both oral and subcutaneous administration of Al (Klein, 1990).

3. The toxicity of Al

The toxic effect exerted by Al on plants was studied by Johnson et al. (1984) who found that Al present in the soil in undissolved form may become toxic primarily if the soil undergoes acidification. This may occur as a result of e.g. the acid rain.

Aluminium exerts its toxic effect by binding to the nuclear DNA and by disturbing the plant's metabolism (Matsumoto et al., 1977).

It may be toxic to fish and other aquatic organisms (e.g. planktons). In this respect, Driscoll et al. (1980) observed that the fluorine salts of Al have lower toxicity.

In mammals, Al may exert its toxic effect in three different ways: (1) locally, e.g. in the intestinal tract; (2) by damaging the lungs (e.g. during continuous inspiration); (3) through a systemic action (after absorption and in the case of oral administration).

An important systemic effect of Al is that after oral ingestion it reduces the absorption and utilization of other elements (e.g. Sr, F, Fe, P and Ca). Owing to this property, it can be used as an antidote in F toxicosis. In uraemic patients Al diminishes the absorption of P. Aluminium compounds impair the absorption of cholesterol and, after binding to pectin, that of lipids (Mertz, 1986).

Free Al inhibits the effect of acetylcholine, thus adversely affecting intestinal motility, the emptying of the stomach and causing passage disturbances (Hava and Hurwitz, 1973).

The effect exerted by Al on the lungs was first described in 1934 (Filip, 1934). Several observations suggest that the prolonged inhalation of bauxite (Al_2O_3 x 3 H_2O) causes pulmonary fibrosis (Schaver, 1948). Drew et al. (1974) reported granulomatosis of the bronchioles in animals under prolonged exposure to aluminium chlorohydrate.

3.1. The pathomechanism of aluminium toxicosis and the toxic dose of Al

Although Al is known to inhibit several biological and enzymatic processes, the mechanism of Al toxicosis has remained unclear in many respects.

The neurotoxic action of Al can partly be explained by changes induced by it in the structure of tissue proteins and by its interaction with proteins (Siegel and Hang, 1983). Aluminium increases O_2 uptake and enhances acetylcholinesterase activity through the succino-dehydrogenase-cytochrome system (Patocka, 1971). *In vitro* experiments have shown that Al induces the proliferation of neurofilaments in neuroblast cell cultures. It reduces the RNA content of cells and at increasing protein content it enhances leucine incorporation with a simultaneous decrease in acetylcholine activity (Miller and Levine, 1984). Al causes local damage of the nervous tissue as a direct effect of inoculation (Bizzi et al., 1984).

Forming deposits in the bones, Al inhibits the calcification of osteoid tissue (Maloney et al., 1982) whilst accumulating in the mitochondria of osteoclasts (Cournot-Witmer et al., 1985). It inhibits phosphatase activity (Lieberherr et al., 1982). From these effects it is easy to understand why Al toxicity often manifests itself only as secondary P deficiency. When the feed was supplemented with P or P was inoculated intramuscularly in the form of PO_4, the signs of Al toxicosis did not develop in chickens even if the birds had previously shown motor disturbances (Deobald and Elvehjem, 1935; Thurston et al., 1972).

Primarily the soluble salts (acetate, chloride, nitrate and sulphate compounds) of Al are toxic, while e.g. its oxides and phosphates have lower toxicity (Street, 1942). This is due to the fact that PO_4 salts prevent secondary P deficiency and that $AlSO_4$ forms a complex precipitate with dietary phosphorus in the intestine, thus diminishing the effect of Al. At the same time, high F content of the feed lowers the retention of already utilized Al.

Ruminants are less susceptible to Al toxicosis as the organic anions present in the rumen form a complex with Al (Thompson et al., 1959).

As regards its presumed anaemia-producing effect, Al was shown to inhibit the activity of ceruloplasmin (ferroxidase) and, after binding to transferrin, to disturb Fe metabolism (Huber and Frieden, 1970).

In the most severe form of toxicosis Al inhibits glycolysis and phosphorylation (Thompson et al., 1959).

Conflicting views have been published in the literature as regards the amount of Al retained in the tissues. Table 1 shows the Al content of the tissues in some species of animals. Aluminium content may vary widely by organ and by animal species (Valdivia et al., 1978).

Table 1

Mean physiological Al concentrations measured in the tissues of some species

Species	Organs						Author
	Liver	Kidney	Skeletal muscle	Myo-cardi-um	Brain	Bone	
Cattle[1]	7.6	4.5	3.8	-	6.4	-	Mertz, 1986
Sheep[1]	2.8	2.9	2.6	-	4.5	-	Mertz, 1986
Fattening lamb[1]	2.6	2.4	2.3	-	3.7	-	Valdivia et al., 1982
Steer[1]	7.6	4.5	3.8	-	6.4	-	Valdivia et al., 1982
Rat[1]	15.5	8.6	0.4	10.8	7.1	702.0	Mertz, 1986
Man[2]	4.1	-	1.2	1.0	2.2	3.3	Alfrey et al., 1980

[1] = mg/kg wet matter; [2] = mg/kg dry matter

The maximum tolerable level of Al depends on two main factors, the P content of the diet and the solubility of the Al compound. Experiments conducted with soluble Al salts in calves (Bailey and Valdivia, 1977) revealed that not even feeds containing Al at levels as high as 1,200 ppm caused toxicity. Nor did a diet containing 1,215 g Al per kg prove toxic when fed to sheep. On the basis of these results, the Al dose still tolerable by cattle and sheep was set at 1,000 ppm.

Chickens tolerated a diet containing 486 ppm Al without any adverse effects (Cakir et al., 1978). In contrast, others (Storer and Nelson, 1968) found that the body mass gain and feed utilization of broiler chickens were markedly impaired already after the feeding of a diet containing 500 ppm Al. Turkeys fed a diet containing 486 g/kg Al showed no adverse effects either.

In monogastric animals, the tolerance level of Al taken up in the form of soluble Al compounds was set at 200 ppm.

3.2. Specific effects of Al on some organs. Clinical signs and pathological lesions

Bone lesions

The bone lesions caused by Al have most thoroughly been studied in humans since the 1970's. Osteopathies manifesting themselves in bone fractures and ostealgia were accompanied by defective bone formation and, in the most severe case, osteomalacia. The latter was found in connection with haemodialysis treatment of patients with severe renal failure if the dialysis fluid was contaminated with Al (Platts et al., 1977). In such cases, substantial amounts of Al accumulated in the bones. A similar disease was described also after the application of phosphate-binding gel (Andreoli et al., 1984). The lesion was produced by Al that accumulated in the osteoid tissue indirectly, as a result of repeated Al action and through an inhibitory effect exerted on the hormone production of the parathyroid gland. Vitamin D deficiency may facilitate the development of osteopathies either directly or, even more so, indirectly, through depressed production in nephropathic patients. Bone abnormities may develop in neonates, especially in premature infants, fed exclusively through the parenteral route using an infusion contaminated with Al. Newborn infants normally retain more Al due to the reduced glomerular filtration.

Brain lesions

The prolonged administration of Al-containing fluids may lead to the development of severe encephalopathies with simultaneous Al accumulation (Alfrey, 1986). After a similar intervention, Bakir et al. (1986) reported acute encephalopathy which developed and led to death within one month. Histologically the degeneration of neurofibrils could be observed, and aluminium accumulation was seen mainly in the grey matter (Alfrey et al., 1976; Wenk and Stemmer, 1983).

Liver lesions

Hepatopathy accompanied by cholestasis develops mainly in newborn infants. The condition, which is characterized by mild periportal inflammation, cholestasis and fat deposition, may develop into cirrhosis. In addition to arrested intestinal activity, the glucose and amino acid constituents of the given fluid are also involved in that factorial disease (Balistreri

et al., 1986). In older children given a fluid containing casein as protein source, the Al concentration of the liver underwent a 20-fold increase (Klein et al., 1982*a*). In contrast to its experimentally demonstrated active involvement in the development of bone and brain lesions, no direct role of Al in the production of liver lesions has been demonstrated, except the observation suggesting that it causes lysosomal damage (Galle and Giudicelli, 1982).

4. Study of the effect of aluminium in animal experiments

4.1. Experiments with poultry

Storer and Nelson (1968) studied the effect of different Al compounds on the performance of chickens. The aim of their studies was to check the well-known fact that certain aluminium compounds poorly, or not at all, absorb from the intestinal tract (Jones, 1938).

Properly grouped and housed day-old cockerel chicks were used in the experiments. The Al-free basic diet was supplemented with 0.3% phosphorus (monosodium-orthophosphate) and 0.6% calcium ($CaCo_3$). In addition to this diet, the experimental groups received water-soluble (aluminium acetate, aluminium chloride, aluminium nitrate and aluminium sulphate) and insoluble (aluminium oxide and aluminium phosphate) Al compounds at different concentrations. Feed and drinking water was provided *ad libitum*. The experimental feeding lasted 2–3 weeks. The results can be summarized as follows. (*i*) If water-soluble compounds were added to the feeds to give an Al content of 0.5%, the mortality rate of chickens in all the four experimental groups came close to, or reached, 100%. (*ii*) Addition of aluminium chloride and aluminium sulphate to the diet at lower concentrations (0.05–0.40%) adversely affected the body mass gain, feed conversion efficiency and bone calcification, but did not cause deaths. (iii) The feeding of water-insoluble aluminium oxide and aluminium phosphate compounds in a quantity to give 0.025–1.60% Al concentration did not alter the chickens' performance.

From these results they conclude that the feeding of Al-containing diets impairs the performance of chickens only if the feed is supplemented with aluminium compounds highly soluble in water.

Lipstein and Hurwitz (1981) studied the effect of three *Micractinium* alga species mixed to the diet of broilers and laying hens as protein source. Their aim was to determine if the feeding of alga cultures relatively rich in aluminium (3.9–5.3 %; Table 2) and thus markedly increasing the Al content of the diet causes any changes (and if yes, what changes) in broiler and laying hen flocks. They used day-old chicks and layers at the peak of egg production.

Table 2

Chemical composition of three samples of *Micractinium* algae (Lipstein and Hurwitz, 1981)

Parameter (%)	Alga samples		
	A[a]	B	C
Total protein (N X 6.25)	38.00	39.50	38.80
Total lipid	4.90	5.60	3.70
Fibre	1.00	0.50	0.10
Ash	19.60	21.80	22.50
Phosphorus	2.19	3.00	2.69
Calcium	0.65	0.67	0.57
Aluminium	5.30	3.90	5.00

[a] This sample was used in the broiler and layer trials

The diets fed to three groups of broilers and groups of laying hens were supplemented with algae at a rate of 3.9–15% to give a high dietary Al content. In addition to continuously monitoring some content values of the diets, the birds' behaviour, protein utilization and production were observed. The main findings were as follow: (*i*) Only negligible changes occurred in the amino acid content of diets supplemented with alga meal. (*ii*) The inorganic P content of the chickens' blood plasma decreased proportionally to the Al concentration. (*iii*) The feeding of alga meal to 7-week-old broilers at 6% of the diet had no adverse influence on body mass gain and feed utilization. (*iv*) The feeding of alga meal at 9% of the diet reduced the feed intake; which resulted in decreased fat accumulation in the abdominal cavity in the final period of fattening. (*v*) Elevated alga meal con-

tent of the diet fed to laying hens led to dropped egg production, lower egg mass, reduced feed intake and impaired specific feed utilization. (*vi*) Because of the high Al content of alga meal, dietary dicalcium phosphate concentration had to be increased (doubled or quadrupled) proportionally with the increase in the concentration of the alga meal added.

In another trial conducted on 120 chickens, Lipstein and Hurwitz (1982) attempted to accurately determine the amount of phosphorus needed to compensate for the substantial amount of aluminium taken up with the alga meal. The birds were divided into 10 groups. Four groups received no alga meal. The remaining six groups were fed 10% alga meal containing 5% Al and 2.4% P. The P content of the diet fed to the different groups varied between 0.38 and 1.42%, in increasing order. The clinical and bone examinations revealed that, when feeding alga meal, 0.76 g phosphorus must be given for every g of Al in 1 kg of diet in order to prevent defective ossification and hypophosphataemia.

Hussein et al. (1990*a*) studied the interactions of Al, Ca and P and the effect exerted by dietary aluminium sulphate on the Ca and P metabolism in day-old chickens. In one of the two experimental groups they studied the interactions of Al, Ca and P and their effect on the chickens' growth and feed utilization, while in the other group the physiological effects of aluminium sulphate were investigated.

In the first experiment, four groups of chickens received different amounts of Ca and P as follows: 0.90% Ca and 0.45% utilizable P, 0.90% Ca and 0.78% P, 1.80% Ca and 0.45% P, and 1.80% Ca and 0.90% P. The diet of the control group contained 0.90% Ca and 0.45% P. On days 22–439 of the feeding trial the basic diet of the experimental chickens was supplemented with 0.392% Al given in the form of aluminium sulphate.

In the second experiment, the control diet was fed (0.90% Ca and 0.45% P) either alone or supplemented with 0.2% Al (as aluminium sulphate) or 1.06% sulphate (as potassium sulphate). The birds were housed properly and received feed and drinking water *ad libitum*. The feeding trial yielded the following main results: (*i*) the feeding of Al significantly reduced the body mass gain, the feed intake, the gain/feed ratio, the inorganic P content of the plasma, the mass and ash content of the tibia in all groups except the one fed 1.8% Ca and 0.90% P. (*ii*) In some cases rickets could be observed in the experimental chickens fed 0.392% Al (Table 3). (*iii*) Al significantly reduced the Zn content of the plasma up to day 21 of the

feeding period, except if the feed contained 1.8% Ca and 0.9% P. (*iv*) The phosphorus content of the plasma and the ash content of the tibia increased if the utilizable P content of the diet was higher, even if Al was present. (*v*) The administration of Al adversely affected the feed intake and the body mass gain throughout the trial (49 days).

Table 3

The effect of dietary Al, Ca and available phosphorus (P_{av}) on growth performance and bone mineralization[1] (Hussein et al., 1990*a*)

Dietary treatment			Weight gain	Feed intake	Gain:feed ratio	Tibia weight	Tibia strength	Tibia ash
Al (%)	Ca (%)	P_{av} (%)	(g)	(g)	(g/g)	(g)	(kg)	(%)
0	0.90	0.45	593	822	0.724	1.93	5.58	47.4
0	0.90	0.78	560	758	0.739	1.96	6.92	45.9
0	1.80	0.45	510	684	0.745	1.60	5.84	43.6
0	1.80	0.90	587	811	0.725	2.10	6.71	50.5
0.392	0.90	0.45	101	203	0.487	0.38	1.78	32.1
0.392	0.90	0.78	120	227	0.487	0.41	2.46	39.6
0.392	1.80	0.45	76	200	0.382	0.37	1.83	27.4
0.392	1.80	0.90	178	288	0.618	0.69	4.83	46.3

[1] Each diet was fed to three groups of 10 chicks for 21 days. Tibiae (two per replicate group) were fat-extracted and dried before weighing, breaking and ashing

In the second experiment the administration of 0.2% Al reduced the body mass gain, the feed intake, the gain/feed ratio, and the plasma phosphorus level. No such effects could be observed upon the administration of potassium sulphate, indicating that the above-mentioned toxic effect of aluminium sulphate was due to Al rather than to the sulphate.

Sooncharernying and Edwards (1990) studied the aluminium and microelement, more specifically iron, content of poultry feeds. They found that a concentrate consisting of 13 components contained also iron and aluminium, though in varying amounts (Table 4). In their experiment conducted on broiler chickens, they supplemented the diet with 0, 125, 250

and 500 mg Al and with 0, 250 and 1,000 mg Fe. Besides Ca and P retention, they monitored the chickens' body mass gain and feed utilization.

Among other things they found that Al administered at 250 mg/kg of feed impaired feed utilization while that given at a dose of 500 mg/kg of feed already reduced the body mass and the quantity of bone ash as well. The same was observed when Fe was fed at a rate of 1,000 mg/kg of feed. The increased P level of the diet diminished the harmful (toxic) effect of Al and Fe (Tables 5 and 6).

In a feeding trial conducted with broiler chickens, Hussein et al. (1990*b*) supplemented the diet with 0.0, 0.196 or 0.392% Al (given as aluminium sulphate), while the utilizable phosphorus content of the diet was also varied (0.45, 0.68 and 0.78%).

In experiment 1 they found that the inorganic P content of the plasma significantly rose parallel with increasing dietary P content. If Al was also given, plasma inorganic P content decreased. The body mass gain and feed intake of broiler chickens given increasing dietary Al concentrations considerably decreased by day 21. At the same time, these variables remained unchanged if dietary P content was raised. In chickens given 0.196% Al body mass gain decreased by 39% and feed intake by 34%, while in chickens fed 0.392% Al the respective decreases in these two parameters were 73% and 66%.

In experiment 2 the diet was supplemented, besides the above Al doses, with different amounts of P and Ca. In the 21-day feeding trial the following main observations were made: (*i*) Al supplementation reduced the body mass gain; (*ii*) the P content and total Ca, Zn and Mg content of the plasma decreased; (*iii*) the tibia became more brittle and its mass decreased; (*iv*) as a result of the increasing P content (0.9–1.8%) the body mass gain, the P content of the plasma and the mass of the tibia increased; (*v*) increasing dietary P content (0.45–0.90%) resulted in lowered plasma P concentration and elevated total Ca content in the plasma; (*vi*) by increasing dietary P content, the adverse effect otherwise exerted by Al on growth could be eliminated.

Table 4

Trace element composition of feedstuffs commonly used for poultry (Sooncharernying and Edwards, 1990)

Feedstuffs	No. of samples	Concentration (mg/kg)							
		Al	Cu	Fe	Mn	Mo	Pb	Si	Zn
Yellow corn	3	10 ± 7	1.4 ± 1.1	65 ± 13	8 ± 3	0.08 ± 0.04	1 ± 0.9	0.8 ± 0.6	18 ± 3
		2 – 16	0.1 – 2	57 – 80	5 – 11	0.03 – 0.1	0.1 – 2	0.1 – 1.2	15 – 20
Soybean oil meal	3	60 ± 28	14 ± 5	204 ± 36	40 ± 5	0.36 ± 0.06	3.3 ± 1.2	5.3 ± 2.3	3.55 ± 4
		28 – 82	8 – 17	164 – 234	34 – 0.3	0.3 – 0.4	2 – 4	4 – 8	52 – 59
Dicalcium, feed grade	1	7049	7	6104	293	21	35	209	61
Dicalcium, food grade	1	249	-	155	10	3	18	97	8
Defluorinated phosphate	1	8592	14	6454	203	15	54	666	178
Limestone	1	361	2	363	29	29	27	274	-
Poultry fat	1	2053	6	103	13	1	2	1	38
Trace mineral mix	1	8329	8032	5.72%	11.4%	72	243	156	7.85%
Selenium premix	1	373	14	483	291	3.9	33	237	249
Vitamin mix	1	1558	7	2358	161	1	19	266	48
Meat-and-bone meal	1	175	14	622	37	0.01	13	70	131
Poultry by-product	6	233 ± 46	36 ± 4	3051±724	11 ± 1	0.28 ± 0.4	9 ± 2	38 ± 8	117 ± 6
		151 – 290	31 – 41	2000–4228	10 – 12	0.2 – 0.3	7 – 12	30 – 52	110 – 124
Corn-soy diet	5	166 ± 39	8 ± 1	395 ± 86	64 ± 12	0.2 ± 0.09	3.4 ± 0.5	13 ± 2	74 ± 14
		123 – 217	7 – 10	263 – 500	45 – 77	0.1 – 0.3	3 – 4	11 – 15	65 – 99

Table 5

Effect of supplemental Al and Fe in diets containing 0.55% available P (Sooncharernying and Edwards, 1990)

Supplementation		Body weight 16 days	Gain/Feed	Bone ash	Rickets	Retention (14th day)		
						Ca	Total P	Phytin P
mg/kg		g		%	%	%	%	%
Al	0	382	0.72	38.3	24	52	45	6
	125	372	0.71	38.5	15	53	46	10
	250	364	0.69	38.4	13	55	47	13
	500	358	0.70	37.7	11	57	47	13
Fe	0	372	0.70	38.5	24	49	46	2
	250	375	0.71	38.4	13	54	46	8
	500	380	0.72	38.3	12	55	45	4
	1,000	350	0.68	37.6	14	59	47	27

Table 6

Effect of supplemental Al and Fe in diets containing 0.45% available P (Sooncharernying and Edwards, 1990)

Supplementation		Body weight 16 days	Gain/Feed	Bone ash	Rickets	Retention (14th day)		
						Ca	Total P	Phytin P
mg/kg		g		%	%	%	%	%
Al	0	388	0.73	37.7	14	57	54	28
	125	373	0.70	37.5	27	54	54	34
	250	369	0.69	37.4	43	50	53	32
	500	347	0.69	36.7	41	51	51	29
Fe	0	372	0.71	37.9	48	50	52	24
	250	375	0.71	37.7	28	52	52	25
	500	383	0.72	37.6	19	55	54	28
	1,000	347	0.67	36.1	31	54	54	46

Elliot and Edwards (1991) studied the effect of dietary aluminium and silicon content in broiler chickens. The basal ration of chickens assigned to four experimental groups was based partly on casein and gelatin, partly on soybean groats, and it contained varying amounts of Ca and P. During the 16-day period of observation the following variables were monitored: body mass gain, feed utilization, bone ash quantity, tibial status, Ca, P and phytin phosphorus retention. The main results were as follow: (*i*) the body mass of the two groups of broilers fed a casein- and gelatin-based diet and that of broilers fed a diet supplemented with 4,000 mg Al significantly decreased, and their feed utilization worsened. (*ii*) The quantity of bone ash decreased. (*iii*) Similar observations were made when feeding a diet of lower Al content (500 and 2,000 mg Al/kg of feed), ccntaining Al in the form of aluminium sulphate). (*iv*) Al supplementation reduced the incidence and severity of tibial chondrodystrophic lesions; this effect, however, was always associated with a reduction in body mass gain. (*v*) The rising dietary Al concentration reduced the retention of phosphorus and phytin phosphorus. (*vi*) Silicon supplementation did not diminish the adverse effect of Al on the above-mentioned variables. (*vii*) Al seems to have exerted its toxic effect on chickens through reducing P and phytin phosphate retention.

In experiments with broiler chickens, supplementation of the diet with 0.5 and 1.0% sodium-calcium-aluminium silicate did not impair the utilization of Mn, vitamin A and riboflavin, while it moderately impaired that of Zn (Chung et al., 1990).

Hussein et al. (1989*a*) studied and compared the effect of Al fed in the diet in an experiment with 72-week-old Leghorn laying hens. The hens of groups A and B were fed a maize and soybean groats based diet containing 15% crude protein, 3.5% Ca and 0.35% utilizable P *ad libitum*. The feed was supplemented with 0.3% Al added in the form of aluminium sulphate. The hens of group C were deprived of feed for 10 days. Drinking water was provided to all laying hens *ad libitum*. The main results of the experiment were as follow. (*i*) In group C, egg production completely stopped from the 7th day of feed deprivation. (*ii*) By day 15 of the feeding trial egg production gradually ceased also in groups A and B fed a diet containing 0.3% Al. (*iii*) Average body mass decreased in all three groups; the most pronounced decrease occurred in group C. (*iv*) Feed intake decreased considerably (approx. by 60%) in the group fed a diet supple-

mented with 0.3% Al. (*v*) The Al-supplemented diet reduced egg production by day 15 of the feeding trial, while feed deprivation resulted in dropped egg production sooner (already after one week). (*vi*) Al is considered suitable for artificially terminating egg production; however, feed deprivation is considered a more practical and economical method for that purpose.

In another experiment with Leghorn laying hens, Hussein et al. (1989*a*) studied the effect exerted by Al administered in the diet on Ca and P metabolism and on production.

In the first experiment, 0, 0.05, 0.10 or 0.15% Al (given in the form of aluminium sulphate) was mixed to a maize or soybean groats based diet containing 3.3% Ca and 0.5% utilizable P. During the 28-day feeding trial the layers' body mass, feed intake, egg production, and plasma inorganic P content significantly decreased and the tibia became more brittle.

During the second feeding trial, Al was added to the basal ration at increasing rates (0.0, 0.1, 0.2 and 0.3%). As a result of the 42-day feeding of 0.3% Al, the plasma calcium content did not change while the mass and brittleness of the tibia did.

The results of these feeding trials confirmed that the feeding of Al to laying hens at a rate of 0.15–0.30% adversely influenced the layers' P metabolism and egg production.

Rossi et al. (1990) studied the effect of Al feeding on phosphorus utilization in laying hens. In their experiment involving one hundred and sixty 64-week-old Leghorn layers, the basal ration containing 0.5 or 1.0% P was supplemented with varying amounts (0.0, 0.2, 0.4 and 0.6%) Al given in the form of aluminium acetate. Feed and drinking water were provided *ad libitum*. The feeding trial lasted 15 days. The calculated concentration of P in the feeds of different Al content was between 0.03 and 0.98%. The main results derived from the feeding trial are as follow: (*i*) egg production was significantly lower in the two groups fed the lowest amount of dietary P (0.03 and 0.18%, respectively). (*ii*) Twenty laying hens of the group fed 0.03% P laid no eggs at all on the last two days of the feeding trial. (*iii*) Plasma inorganic P content significantly decreased both in the hens fed a low P diet and in those given a diet of high Al content. The negative effect of Al supplementation could be completely prevented by increasing the P content of the diet. This suggests that the observed negative influence is due to the reduced utilization of P caused by

Al. (*iv*) The reduction in egg density caused by P deficiency could be prevented by Al administration. (*v*) Maximum feed intake and egg production could be observed if the dietary P content was between 0.33 and 0.98%.

In three experiments involving several hundred laying hens assigned to three groups, Roland (1991) compared the effect exerted by the expressly ion-exchanger sodium-aluminium-silicate (ETHACAL) on egg quality with that of other aluminium compounds. The feed intake and egg production of the layers and the mass of eggs were monitored. The layers' feed based on maize and soybean groats was supplemented with varying concentrations of the compounds mentioned above

In the first experiment 0.0 (control) and 1.5%, in the second experiment 0.0, 0.75 and 1.5%, while in the third experiment 0.0, 1.0 and 0.58% sodium-aluminium silicate was mixed in the basal ration. In the third experiment, one group received aluminium sulphate while another aluminium phosphate supplementation to give dietary Al concentrations of 0.148 and 0.101%, respectively.

Egg production, egg mass and feed intake were monitored during the feeding trial. The trial yielded the following results. (*i*) The feeding of diets containing sodium-aluminium silicate resulted in a significant increase in egg density in all experimental groups. (*ii*) The density of eggs did not change in the case of layers fed aluminium sulphate (0.148% Al) and aluminium phosphate (0.101% Al). (*iii*) The authors conclude that egg density was increased by Al from sodium-aluminium sulphate disposed to ion exchange.

Similar results were obtained by other authors (Pethiere et al., 1990) in experiments conducted on 40- to 60-week-old laying hens. Among other things they found that it is advisable to supplement synthetic diets containing sodium-aluminium silicate with adequate amounts of phosphorus, as otherwise feed utilization becomes impaired and egg production as well as the mass of eggs decrease.

In five feeding trials involving a total of 2,160 laying hens aged 38-80 weeks, Roland (1988) studied the effect of sodium-aluminium silicate administered alone or in combination with various chlorides. The feeding trial lasted 6–8 weeks. The administration of the different compounds, the egg production and the feed intake are summarized in tables. The main results of the feeding trial were as follow. (*i*) In all but one experiment (no. 2), the feeding of diets containing sodium-aluminium silicate resulted in

increased egg density. (*ii*) The feeding of 0.75% sodium-aluminium silicate increased the density of eggs by 1–3 units. No sodium adjustment was needed for the above compound to exert its favourable effect.

Hussein et al. (1988) conducted three experiments on Japanese quail to compare the effect of Al on egg production. A standard basal ration containing 2.5% Ca and 0.35% P was supplemented with 0.3% Al (in the form of aluminium sulphate) in one group and with 1.5% Zn (given as zinc oxide) in the other. The main findings were as follow. (*i*) Egg production decreased both in groups fed an Al-supplemented and in those given a Zn-supplemented diet. (*ii*) The solidity of the egg shell decreased. (*iii*) Al supplementation of the diet significantly lowered the inorganic P level of the plasma by day 5 of the trial. (*iv*) Al administered at high concentrations lowered egg production in quails. When present at sufficient quantities, it terminated egg production through an adverse influence exerted on feed intake, P metabolism, or both.

4.2. Experiments with ruminants

Allen et al. (1986) conducted feeding trials in fifty 8- to 10-year-old milking beef cattle (average body mass: 430 kg). The animals were fed a diet containing 200 μg/kg Al or a diet supplemented either with aluminium citrate, citric acid and earth or with earth and citric acid. After the addition of the latter components the diet contained 1,730, 1,830 and 1,935 μg/g Al. The addition of soil resulted in elevated dietary Mg and Fe content. The main results of the feeding trial were the following: (*i*) The pH of the rumen content changed depending on the chemicals added; (*ii*) The rumen content of cattle fed the basal ration contained 800 μg, while that of animals fed a diet contaminated with earth and treated with citric acid contained 2,910 μg Al per 1 g of dry matter. (*iii*) The Mg and inorganic P content of the serum decreased. Animals fed a diet supplemented with aluminium citrate had serum P concentrations of 3.8 and 6.8 mg/dl, respectively. (*iv*) The treatment caused no significant changes in the Ca, P and Zn content of the bone ash; however, Mg concentration moderately decreased in animals fed a diet supplemented with aluminium citrate. (*v*) The results indicate that in dairy beef cattle Al intake can change the metabolism of Mg, Ca and P; that effect, however, depends also on the aluminium source and the amount of Al ingested.

Valdivia et al. (1978 *a, b*) conducted feeding trials with 34 feedlot cattle of 226 kg average body mass. The bulls were assigned to four groups and fed a diet supplemented with 0, 300, 600 and 1,200 mg Al (given as aluminium chloride) per kg of feed for 84 days, in order to monitor the effect of Al supplementation on production and on certain physiological variables. The feeding trial yielded the following main results: (*i*) The elevation of dietary Al content did not exert a significant effect on feed intake, body mass, feed utilization, and blood plasma P, Ca, Mg and Al concentration. The haemoglobin concentration and packed cell volume (haematocrit value) did not change either. (*ii*) As compared to the control animals, the Al, Fe, Mn, P and Ca content of the liver, kidney, back muscle and brain did not change (Table 7). (*iii*) Zn content of the liver and kidney increased as a result of Al supplementation. (*iv*) Even the feeding of diets containing Al at a concentration as high as 1,200 mg/kg failed to alter the animals' performance.

Neathery et al. (1990*a*) studied the effect exerted by different amounts (0.0–0.20%) of Al and normal as well as subnormal concentrations (0.0–0.22%) of P added to the normal ration of 72-day-old calves of 64 kg average body mass on Mg metabolism. The diets fed to the four groups of animals can be characterized as follows:

Group	Al concentration	P concentration
1	low	normal
2	low	low
3	high	normal
4	high	low

Table 7

Effect of dietary aluminium content on the mineral composition of four organs in steers[a] (Valdivia et al., 1978*a*,*b*)

Dietary aluminium	Tissue minerals (mg/kg fresh tissue)[a]							
	Al	Fe	Zn	Cu	Mn	P	Ca	Mg
				Liver				
0	7.6	28.2	19.4	59.1	3.00	1,502	28.2	98.0
300	5.7	40.9	23.6	33.1	2.87	1,841	30.8	122.8
600	10.0	30.5	21.0	48.5	3.06	1,683	29.3	111.7
1,200	11.2	35.7	26.6	53.5	3.30	1,989	31.0	130.0
				Kidney				
0	4.5	25.5	9.1	4.88	0.74	1,004	35.0	196.4
300	4.1	31.6	10.1	5.35	0.74	1,113	42.6	119.9
600	6.0	22.6	7.3	5.00	0.76	890	32.8	164.5
1,200	5.4	32.5	11.7	5.53	0.79	1,242	42.2	238.4
				Muscle				
0	3.8	15.4	17.8	0.89	0.11	1,482	37.1	224.8
300	3.9	15.7	19.7	0.85	0.10	1,454	34.7	235.5
600	5.4	15.8	22.0	0.84	0.11	1,568	34.7	235.8
1,200	4.7	14.1	17.6	0.75	0.13	1,399	37.8	210.7
				Brain				
0	6.4	23.1	8.2	21.9	0.41	2,173	57.3	117.5
300	7.6	20.8	10.5	20.8	0.40	2,396	92.8	132.4
600	5.5	19.1	10.1	23.3	0.37	2,114	76.1	120.5
1,200	7.7	24.5	9.8	20.6	0.38	2,222	67.2	123.7

[a] Means represent data from six steers

By laboratory assay, the basal (control) ration was found to contain 0.132% P, 0.021% Al and 0.17% Mg. The most important results of the feeding trial were as follow: (*i*) Al supplementation did not affect the Mg concentration of the serum. (*ii*) The Al–P interaction affected serum Mg concentration. (*iii*) In calves fed an Al-supplemented diet the absorption of Mg dropped approximately to one-fifth the normal value, and the amount of Mg excreted in the urine also decreased. (*iv*) if a diet of normal P level was fed, more Mg was incorporated into the calves' bones (the tibia). (*v*) Al supplementation of the diet adversely affected the Mg metabolism of calves.

In an experiment using young, 64-day-old calves Neathery et al. (1990*b*) studied the effect of Al and P on Zn metabolism. Both Al and P were administered at two different concentrations (Al: 0.0 and 0.20%; P: 0.0 and 0.22%) also in this experiment.The treatment groups were also similar to those used in the above trial.

Group	Al concentration	P concentration
1	low	normal
2	low	low
3	high	normal
4	high	low

Assay of the basal ration revealed 0.132% P, 0.74% Ca, 0.021% Al and 59 mg Zn per kg of feed. The feeding trial lasted 7 weeks. The main results of the experiment were as follow: (*i*) Zn absorption was more efficient if a low-P diet was fed. (*ii*) Al supplementation impaired the absorption of Zn. (*iii*) In the liver, kidney, spleen, myocardium and testicles of animals fed a low-P diet a higher Zn concentration was measured than in the corresponding organs of calves fed a diet of normal P level. (*iv*) Calves fed a diet of high Al content had lower Zn concentrations in the pancreas, myocardium, skeletal muscle and testicles. (*v*) When diets of subnormal P content were fed, Fe concentration of the liver and kidneys and Mn concentration of the liver increased while Cu concentration of the tibia and skeletal muscles decreased. (*vi*) The results indicate that the low P and high Al content of the diet fed to calves affects the animals' Zn metabolism, though only to a limited extent.

Schenkel et al. (1986) monitored the concentration of Al in the blood serum and, after slaughter, in the liver and kidneys of feedlot cattle and dairy cows fed a diet of known Al content. The Al content of the blood serum was found to be 6–9 ng/ml, that of the bones (calculated for wet matter) was 325–1,886 ng, while the liver and the kidney contained 100 and 120 ng Al per g, respectively. In three dairy cows fed 10 g Al daily (in the form of aluminium sulphate) over a period of 15 weeks, the Al content of the blood serum gradually increased to 4–5 times the initial value (from 10 to 40–50 μg/ml), then returned to the baseline level after Al administration had been discontinued.

In their experiment involving eighteen 60-day-old wethers, Allen et al. (1991) studied the effect exerted by aluminium citrate and citric acid administered in the diet on the mineral element content of some tissues. The animals were assigned to three groups and fed the following diets. The diet fed to animals of group 1 (control) contained little Mg (0.12%) and much K (2.87%) per kg of dry matter. Group 2 was fed a diet supplemented with 2,000 μg Al per g, given in the form of aluminium citrate. The diet fed to wethers of group 3 was supplemented with citric acid to give a citrate content identical with that fed to group 2 animals. The compounds were administered through a rumen cannula, in two parts daily. Numerous organs of the animals slaughtered after day 60 of the trial were assayed for 12 mineral elements. The concentrations of minerals measured in the different organs were presented in tables. The experiment yielded the following main observations. (*i*) The concentration of Al in the bones, liver, kidneys, spleen and pituitary gland, and partly in the brain, of animals treated with aluminium citrate increased as compared to those treated with citric acid. (*ii*) Mg concentration decreased in the rib bone while Ca concentration in the pancreas, kidneys and parathyroid gland of animals treated with aluminium citrate. (*iii*) In the animals treated with aluminium citrate, the K content of the liver decreased, the Fe content of the kidneys increased and the Zn content of the pituitary gland decreased. (*iv*) The P and Na content of the kidneys and the Mn content of the brain decreased in wethers treated with aluminium citrate. These results demonstrate that Al absorbed and accumulated in the different organs if it was administered as a citrate compound. The administration of citric acid lowered only the Na and Mn concentration of the tissues.

Rosa et al. (1982) studied the correlations among the P, Al and Fe content of the diet and the effects exerted by these elements on the performance of lambs and the mineral content of their tissues. In a 176-day experiment involving 24 growing lambs of 31 kg average body mass, the lambs' basal ration containing 0.17% P, 40 mg/kg Fe and 158 mg/kg Al was supplemented with 0.0 or 0.25% P (in the form of NaH_2PO_4) and 0.0 or 1,450 mg/kg Al (in the form of aluminium chloride). The main findings were the following. (*i*) P supplementation increased while Al supplementation lowered the feed intake (Table 8). (*ii*) P supplementation increased while high Fe and Al supplementation reduced the average daily body mass gain. (*iii*) Additional P also increased the body mass gain and the feed intake if the diet contained much Fe and Al. (*iv*) High Al content of the diet lowered serum P concentration. (*v*) Additional Fe supplementation of the diet increased the haemoglobin content of the blood and the packed cell volume (haematocrit value). (*vi*) High dietary Al concentration increased the Fe content of the liver (Table 9) and the Zn content of the kidneys but lowered the P and Mg content of the kidneys, the bone ash and its Mg content (Table 10).

Table 8

Effect of dietary P and Al level on the body mass gain of fattening lambs[a] (Rosa et al., 1982)

Dietary P, %	Added Al, ppm	Average daily feed intake, kg	Average daily gain, g	Feed/unit gain
		Subtreatment effect		
0.15	0	1.28	109	11.8
0.29	0	1.32	116	11.3
0.15	2,000	0.78	-38	
0.29	2,000	1.08	15	72.1
		Main treatment effect		
0.15	-	1.03	36	-
0.29	-	1.20	66	41.7
-	0	1.30	113	11.6
-	2,000	0.93	-12	-

[a] Each value represents the mean for five lambs in subtreatment groups and for 10 lambs in main treatment groups; feed on dry matter basis

Table 9

Effect of dietary phosphorus, iron and aluminium on liver mineral composition in lambs[1] (Rosa et al., 1982)

Treatment[b]			Mineral concentration (mg/kg dry matter basis)						
P, %	Fe, ppm	Al, ppm	P	Ca	Mg	Zn	Fe	Cu	Mn
0	0	0	8,301 ± 1,333	128 ± 18	422 ± 68	91 ± 8	162 ± 9	296 ± 31	7.4 ± 1.7
0.25	0	0	8,729 ± 843	122 ± 16	407 ± 47	88 ± 7	164 ± 13	312 ± 25	6.7 ± 0.5
0	760	0	9,588 ± 639	126 ± 14	463 ± 52	104 ± 7	1,306 ± 206	360 ± 94	6.5 ± 0.2
0.25	760	0	8,187 ± 1,064	117 ± 10	410 ± 49	105 ± 18	1,091 ± 201	272 ± 43	6.9 ± 1.2
0	0	1,450	8,206 ± 1,102	124 ± 12	474 ± 38	105 ± 11	185 ± 25	361 ± 18	5.8 ± 0.3
0.25	0	1,450	8,357 ± 967	121 ± 15	443 ± 55	88 ± 7	188 ± 50	394 ± 88	5.9 ± 0.5
0	760	1,450	9,530 ± 187	124 ± 7	536 ± 32	112 ± 9	3,652 ± 383	431 ± 101	5.9 ± 0.4
0.25	760	1,450	9,376 ± 1,309	138 ± 14	473 ± 30	103 ± 21	1,057 ± 420	384 ± 45	7.0 ± 0.9

[1] Each value represents the mean of three animals;

[b] basal diet contained 0.17% P, 40 ppm Fe and 168 ppm Al

Table 10

Effect of dietary phosphorus, iron and aluminium on bone composition in lambs[1]
(Rosa et al., 1982)

Treatment (added minerals)[b]			Ash, %	Mineral concentration (% ash basis)		
P, %	Fe, ppm	Al, ppm		P	Ca	Mg
0	0	0	35.9 ± 0.38	16.8 ± 0.25	32.8 ± 0.70	0.72 ± 0.01
0.25	0	0	39.8 ± 0.07	17.1 ± 0.20	33.9 ± 0.21	0.70 ± 0.03
0	760	0	36.5 ± 1.14	17.1 ± 0.18	33.0 ± 0.58	0.68 ± 0.01
0.25	760	0	39.2 ± 0.14	17.2 ± 0.02	34.3 ± 0.26	0.73 ± 0.01
0	0	1,450	34.4 ± 0.98	16.5 ± 0.18	33.6 ± 0.33	0.59 ± 0.02
0.25	0	1,450	38.0 ± 0.10	17.2 ± 0.08	33.8 ± 0.39	0.67 ± 0.01
0	760	1,450	32.2 ± 0.04	16.6 ± 0.06	34.3 ± 0.21	0.56 ± 0.02
0.25	760	1,450	37.4 ± 0.11	17.1 ± 0.08	33.5 ± 0.11	0.66 ± 0.01

[1] Each value represents the mean ± SE of three lambs;

[b] basal diet contained 0.17% P, 40 ppm Fe and 168 ppm Al

In summary, it can be stated that P supplementation of the diet exerted a beneficial effect on the high dietary Al and Fe content. A conclusion useful for the practice is that high dietary Al and Fe concentration increases the P requirement. This is especially important in the case of grazing animals which ingest much Fe and Al with the soil-contaminated feed.

Valdivia et al. (1982) studied the effect of dietary Al and P content on performance, P utilization, and the mineral element content of the tissues in sheep. In a 56-day trial, a diet containing 0.25 and 0.29% isotope P (^{32}P) and supplemented with 0.0 or 2,000 mg/kg Al in the form of aluminium chloride was fed to a total of 20 sheep of 36.7 kg average body mass, assigned to four groups. From the results they drew the following main conclusions: (*i*) The average daily feed intake of the animals was between 1,320 and 780 g, and was influenced by the amount of both Al and P. (*ii*) The plasma P concentration measured at the end of the experiment was higher in the sheep fed a diet of high Al content. (*iii*) The plasma Ca content of animals fed more P decreased. (*iv*) The Mg content of the blood plasma was lower in sheep fed a diet of higher Al concentration. (*v*) Experiments with radiolabeled P revealed that the administration of Al led to impaired P absorption and retention. (*vi*) The dietary Al concentration exerted an influence on faecal P content. (*vii*) High dietary Al concentration impaired Ca absorption. The absorption of Mg was affected neither by Al nor by P. (*viii*) Elevated dietary Al content increased the Al content of the liver, kidneys and skeletal muscles but did not affect that of the brain. (*ix*) High dietary Al content increased the Fe content of the liver and kidneys and the Cu content of the kidneys while it lowered renal Mg content.

4.3. Experiments with rats

In four experiments, Ecelbarger and Greger (1991) studied the effect of dietary citrate content and renal function on the utilization of Al, Zn and Fe in rats. The main observations were as follow. (*i*) The administration of citrate increased Al retention of the bones if the animals' diet contained 1.0 mg Al per g of diet. (*ii*) Increased Al retention was not directly correlated with dietary citrate content, indicating that citrate exerts a systemic effect on the solubility of microelements in the intestine, thereby facilitating their absorption. (*iii*) If the Ca content of the diet was increased approx. four-

fold, the Al concentration of the bones decreased without impairing the rats' growth. (*iv*) A 30% reduction in renal function resulted in a 34% increase of Al retention in the bones if Al was administered parenterally, and in a 13% higher retention of Al given in the diet. (*v*) Excess aluminium ingested with the feed increased the Al content of tissues only by 0.01–0.05%. (*vi*) The Al content of the tissues and the potential toxicity of Al may vary as a function of feed composition and renal function; however, in the case of oral Al intake it is usually very low.

Laske et al. (1986) studied the effect exerted by Al on the activities of some enzymes and on the cells of certain organs in healthy rats and rats suffering from renal failure, by the intraperitoneal injection of $AlCl_3$ solution for several weeks. Electron microscopic examination of the liver, spleen and kidney of the euthanatized rats showed that the most severe pathological lesion at cellular level was the lysosomal damage. The severity of pathological lesions depended on the Al dose administered.

Aluminium parenterally administered to rats at a dose rate of 10 mg/kg body mass was found to reach uniform distribution in the blood between the red blood cells and the blood plasma (Van der Voet and De Wolf, 1987).

4.4. Other observations

Chappuis et al. (1991) analyzed 12 solutions serving for parenteral nutrition for Zn, Se, Cu and Al content. Slight contamination was found for almost all the solutions examined. Four out of the 12 solutions contained Al in non-negligible quantities: such contamination may pose a risk especially for newborn babies.

Sperber et al. (1991) observed that antacid preparations containing aluminium hydroxide have a serum cholesterol and lipoprotein-cholesterol lowering effect. According to Sperber et al. (1988) this effect may manifest itself already after one-month therapy.

Guida et al. (1991) studied the effect of Al on *Escherichia coli* bacteria, and found that the bacterial growth inhibiting effect of $AlNO_3$ greatly depended on the pH of the medium. Bacteria were the most sensitive at pH 5.4. Electron microscopic examination revealed that Al was intracellularly bound by the microorganisms.

Ming-Ho Yu et al. (1989) tested the blood of 96 persons exposed to F air pollution in the vicinity of aluminium works. Creatinine concentration was found decreased and amylopeptidase activity increased, while total cholesterol level and serum SGOT, SGPT, ALP and LDH activities did not change considerably.

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COMPLEX STUDY OF THE PHYSIOLOGICAL ROLE OF ALUMINIUM
II. ALUMINIUM TOLERANCE TESTS IN BROILER CHICKENS

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Aluminium (Al) tolerance tests were carried out in 8x24 (a total of 192) broiler chickens pre-reared for 17 days. Chickens of the control group were fed a standard poultry grower diet *ad libitum*, while those in six experimental groups received a diet supplemented with different (200, 500 and 1,000 mg/kg of feed) doses of Al supplied in the form of $AlCl_3$ and, in two cases, with the same dose of P added to the feed in the form of MCP, up to 52 days of age. Chickens of one group were fed a diet containing 3,000 mg Al per kg of feed for 70 days. The acidic pH of $AlCl_3$ solution sprayed onto the feed was neutralized by adding a sufficient amount of $NaHCO_3$ solution in all but one group. The birds' health status was monitored regularly throughout the feeding trial. Their body mass gain and feed consumption were recorded weekly. At the end of the trial, 3–10 chickens per group (a total of 40 birds) were exsanguinated, subjected to gross pathological examination, and samples were taken from 9 organs (liver, kidney, skeletal muscle, myocardium, brain, spleen, testicle, lungs and tubular bones) for light and electron microscopic examination and for the regular determination of 8 elements (Ca, P, Mg, Zn, Fe, Mn, Cu, Al). In addition, samples were taken from the organs of 2–5 chickens per group for analysis for additional 6 elements (Mo, Co, Ni, Sr, Na, K). From the results obtained the following main conclusions were drawn: (*i*) With the exception of four birds culled during the trial, all chickens remained symptomless throughout, and all chickens except those fed a diet supplemented with 3,000 mg/kg aluminium developed properly. (*ii*) The body mass of chickens in groups fed a diet supplemented exclusively with Al decreased moderately (by 67, 69 and 88 g, respectively), depending on Al concentration of the diet. Chickens fed a diet containing 3,000 mg/kg Al showed a very substantial (621.3 g; 32%) decrease in body mass Phosphorus supplementation did not markedly affect the body mass gain. (*iii*) Specific feed utilization was satisfactory (2.100–2.210 g/kg body mass). The very poor feed utilization of chickens subjected to the heaviest Al load can be attributed to the temporary dis-

turbance of acid-base balance caused by the non-buffered $AlCl_3$ solution. (*iv*) At the end of the feeding trial the most pronounced accumulation of Al was found in the liver (average concentration: 3.37 mg/kg) and in the bones (55.97 mg/kg) as compared to the controls. The changes in Al accumulation in the other organs followed similar trends. In the group subjected to an Al load of 3,000 mg/kg, the Al content of the bone ash markedly increased while its Ca and Mg content decreased. (*v*) Of the results of correlation calculations performed for 7 elements per organ, the strong positive correlation found between P, Ca, Mg and Zn and the negative correlation established between P, Mg, Cu, Ca and Al content of the bone ash should be emphasized. At necropsy, in several of the birds subjected to a higher Al load the liver was a paler brownish red in colour and the gallbladder was usually markedly dilated. (*vi*) Microscopic examination of the liver revealed bile duct proliferation, solitary hepatocyte necrosis and nuclear lesions indicative of a toxic effect. By electron microscopy, partial degeneration of hepatocytes, dilatation of the endoplasmic reticulum, disintegration of mitochondrial structure, diffuse interstitial oedema of the myocardium, and myofibrillar damage could be observed. In the lumen of the seminiferous tubules only few germinal epithelial cells were seen beside the Sertoli cells which showed lesions indicative of a toxic effect. In the metaphyses and diaphyses of the tubular bones of chickens fed a higher Al dose many inactive osteoblasts showing regressive nuclear lesions could be detected, together with a decrease in the number and size of the bone trabeculae.

Key words: Aluminium load, Al accumulation, body mass change, gross and microscopic lesions, broiler chickens

Aluminium (Al), which is the third most abundant element in the earth's crust, occurs only in traces in biological organisms (Hem, 1986). As a result of certain technological processes and first of all the acid rain, its concentration in freshwaters and drinking water is increasing (Miller et al., 1984). Aluminium can be detected in relatively low but steadily increasing concentrations in plants grown on acidic and clayey soils.

Aluminium enters the organism of animals living under normal conditions via two different routes (via the digestive tract and through the lungs) in quantities that may reach 2–5 mg daily (Alfrey et al., 1980). Its accumulation in different organs depends on numerous factors (Slanina et al., 1984; Long et al., 1980; Alfrey et al., 1980; Godsall et al., 1984).

Although no uniform attitude has been reached yet concerning the essentiality of Al, the increased interest taken in its biological role and the research results obtained in the past two decades have provided us with a closer insight into this problem. The negative effect of Al on the division of

plant cells, on plant growth, and on the absorption and metabolism of other elements such as Mn, P and Mg (Taylor and Foy, 1985) and its several favourable effects exerted on the vital processes of animals have been demonstrated. Of the latter effects, the role of Al in the reaction between cytochrome C and succinyl dehydrogenase and consequently in protein metabolism (Sternweis and Gilman, 1982), in the development of the immune response and in the intermediary metabolism of triglycerides (Sugawara et al., 1987) has been established.

The observations on interactions between Al and other elements and on the cytopathic (toxic) effect of Al entering the organism in large amounts and accumulating in different organs are remarkable (Jeffery et al., 1987). These observations concerning the absorption, metabolism, physiological effects, accumulation within the organism and excretion of Al, together with the results of Al feeding trials conducted in some animal species, are reported in the accompanying paper (Bokori and Fekete, 1993). The results of Al feeding trials in broiler chickens are reported in this work.

Studying the absorption and physiological effects of different Al compounds mixed in the diet, Storer and Nelson (1968) found that the feeding of 0.5% Al in the form of water-soluble aluminium chloride or aluminium acetate resulted in a mortality close to 100% in broiler chickens. The feeding of Al at a dose rate of 0.05–0.40% adversely affected the body mass gain, feed utilization and bone calcification. At the same time, if Al was fed at a dose rate of 0.25–1.6% in the form of water-insoluble aluminium oxide and aluminium phosphate, it did not alter the performance of broiler chickens.

Others (Lipstein and Hurwitz, 1981) mixed in the broiler chickens' diet an alga meal containing 0.3–3.9% Al. The feeding of a diet containing 6% alga meal did not have adverse consequences, while that of a diet containing 9% alga meal reduced the feed intake. From the results of a trial conducted with another group of chickens, Lipstein and Hurwitz (1981) concluded that for every gram of aluminium included in the diet 0.76% phosphorus supplementation must be applied to prevent ossification disorders and hypophosphataemia.

In a feeding trial conducted with day-old chickens, Hussein et al. (1990*b*) studied the interactions of Al, Ca and P and the physiological effect of aluminium sulphate. If between day 22 and 49 of the trial the diet of different Ca (0.9–1.8%) and P (0.4–0.9%) content was supplemented also

with 0.392% Al, the body mass gain, the Zn concentration of the blood plasma, the mass and ash content of the tibia significantly decreased in all groups except the one fed 1.8% Ca and 0.9% P. The feeding of aluminium exerted a negative influence on feed intake and body mass gain throughout. The results of experiments using potassium sulphate demonstrated that the above-mentioned negative effect was due to Al rather than to the sulphate component.

In another feeding trial, the same authors (Hussein et al., 1990*a*) fed to broiler chickens a diet supplemented with 0.0, 0.196 or 0.392% Al and containing utilizable P levels varying by group (0.45, 0.68 and 0.78%, respectively). Increasing Al concentration brought about a proportional decrease in feed intake and body mass gain if the feed was not supplemented with P. Body mass gain decreased by 39% and 73% while feed intake by 34% and 66% in chickens fed 0.196% and 0.392% Al, respectively. If the dietary P level was raised, the adverse effect of Al on growth could not be observed.

The interaction of dietary Al and silicon (Si) was also studied in chickens (Elliot and Edwards, 1991). One of the findings was that the body mass of birds fed a casein and gelatin based diet supplemented with 4,000 mg/kg Al significantly decreased and their feed utilization became impaired. Similar observations were made in chickens fed a diet of lower Al content (500 and 2,000 mg/kg of feed). Increasing dietary Al concentration led to a decrease in the amount of P retained in the bones. Silicon supplementation did not diminish the adverse effect exerted by Al on the above parameters.

According to the results of trials conducted in broiler chickens, supplementation of the diet with 0.1 or 1.0% sodium-potassium-aluminium silicate did not impair the utilization of Mn, vitamin A and riboflavin while moderately reducing the utilization rate of Zn (Chung et al., 1990).

Other authors (Sooncharernying and Edwards, 1990) found that Al fed to broiler chickens at the rate of 250 mg/kg of feed impaired feed utilization while that fed at 500 mg/kg of feed reduced the body mass gain and the quantity of the bone ash. Elevation of the dietary P supplementation level diminished the adverse effect caused by Al.

The present paper reports Al tolerance tests conducted in broiler chickens to study the biological effects and pathological role of that microelement, its accumulation in the organism and its effect exerted on the

metabolism of other elements, as well as to compare the findings with the results of some other trials conducted with broiler chickens so far. The results obtained on the activities of nine enzymes measured in the blood serum of the control and experimental chickens will be reported later.

Materials and methods

Three hundred TETRA-726 meat-type hybrid day-old cockerels were prereared for 17 days. For the Al tolerance tests, 192 cockerels of approximately identical body mass were selected and assigned to eight groups comprising 24 chickens each. (One of the groups contained cockerels chosen from among the approx. 110 g heavier "plus variants".)

The chickens were marked with wing numbers and placed into three-storey broiler rearing batteries in groups of 12 birds in a manner which enabled the elimination of any differences in temperature, relative humidity and illumination between the different levels in the climatized chicken house.

During the 17-day pre-rearing period the chickens were fed a semi-intensive chicken starter diet of the composition and content shown in Table 1, then a semi-intensive broiler grower diet (Table 2), by supplementing the grower diet originally containing 8–10 mg Al with varying amounts of Al (sprayed onto the diet in the form of $AlCl_3$) or Al plus P (added in the form of MCP), as follows.

Groups	Diet fed	Treatments	
		Al* mg/kg feed	P** mg/kg feed
Control	Int. ch. grower	-	-
Al-200	Int. ch. grower	200	-
Al-500	Int. ch. grower	500	-
Al-1,000	Int. ch. grower	1,000	-
Al-P-200	Int. ch. grower	200	200
Al-P-500	Int. ch. grower	500	500
Al-P-1,000	Int. ch. grower	1,000	1,000
Al-3,000	Int. ch. grower	3,000	-

*Al was supplied in the form of aluminium chloride; **P was supplied as MCP. The acidic pH of the $AlCl_3$ solution was neutralized by adding $NaHCO_3$ solution

Table 1

Composition and declared content of poultry starter diet

Composition		kg
Maize		40.0
Wheat		31.0
Extracted soybean, 48%		19.0
Fish meal, 70%		5.0
Energomix–50		1.5
Poultry complete premix		3.5
Total:		100.0
Content		
ME	MJ/kg	12.28
Crude protein	%	20.25
Digestible crude protein	%	17.74
Crude fat	%	3.52
Crude fibre	%	2.39
NaCl (added)	%	0.16
Ca	%	0.94
P (available)	%	0.43
Methionine + cystine	%	0.87
Lysine	%	1.12
Linolic acid	%	1.22
Vitamin A	NE/kg	10,000.00
Vitamin D_3	NE/kg	2,000.00
Vitamin E	mg/kg	11.00

After the end of the feeding trial, four chickens were fed a diet containing 3,000 mg/kg Al for another 70 days.

The diet was provided *ad libitum* from feeding trays, then from feeding troughs, while drinking water was available from valve-type self-waterers. During the feeding trial which lasted up to 52 days of age the chickens' behaviour, feathering and appetite were monitored regularly, the birds were weighed weekly, and their feed consumption was determined. The four chickens that were culled during the feeding trial because of different diseases were subjected to detailed gross pathological examination

and their organs to microbiological examination. At the end of the trial, 3–10 chickens per group (a total of 40 birds) were exsanguinated, subjected to gross pathological examination, and samples were taken from 9 organs (liver, kidney, skeletal muscle, myocardium, brain, spleen, testicle, lungs and tubular bones) for light and electron microscopic examination and for the regular determination of 8 elements (Ca, P, Mg, Zn, Fe, Mn, Cu, Al). In addition, samples were taken from the organs of 2–5 chickens per group for analysis for additional 6 elements (Mo, Co, Ni, Sr, Na, K).

Table 2

Composition and declared content of poultry grower diet

Composition		kg
Maize		45.5
Wheat		28.5
Extracted soybean, 48%		17.5
Fish meal, 70%		4.0
Energomix–50		1.5
Poultry complete premix		3.0
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
Na	%	0.13
NaCl (added)	%	0.18
Ca	%	0.84
P (available)	%	0.62
Methionine + cystine	%	0.72
Lysine	%	1.01
Vitamin A	NE/kg	10,000.00
Vitamin D_3	NE/kg	2,000.00
Vitamin E	mg/kg	11.00
Elancoban	mg/kg	100.00

The content and Al concentration of every batch of the diets used in the trial were checked. The composition of the diets was determined as described by MSZ 6830 (Hungarian Standard). The concentration of mineral elements expressed for dry matter in the diets and in 0.5–1.0 g organ samples processed by the method applied at the Agrochemical and Plant Nutrition Department of the Soil Research Institute of the Hungarian Academy of Sciences was determined by a PERKIN-ELMER 5000 type atom absorption and JY-24 type ICP instrument.

The mineral element content of the different organs was expressed in mg/kg for organs of individual animals or, where the quantity of sample was not sufficient (e.g. testicle, brain) for pooled organ samples of several animals receiving the same treatment. The detailed data were compiled in tables. The average concentrations of different minerals grouped by treatment group were included in two summary tables.

The experimental birds were killed by bleeding and necropsied. For histological examination, samples were taken from the liver, spleen, lungs, testicles, heart and skeletal muscle, brain and the proximal end of the tibiotarsal bone, fixed in 8% neutral formalin, dehydrated and embedded in paraffin. Bone samples taken from the proximal end of the tibiotarsal bone or the femur were decalcified in Ca-EDTA. The sections were stained with haemalum and eosin.

For transmission electron microscopy, approx. 1 mm^3 organ pieces were removed immediately after exsanguination and fixed in 5% buffered glutaraldehyde. The samples were postfixed in 1% osmium tetroxide, embedded in synthetic resin (Fluka), then the semi-thin sections were stained with toluidine blue. Ultrathin sections were counterstained with uranyl acetate and lead citrate. In addition, the sections made from liver, testicle and bone samples were stained also by PAS, Gömöri and Endes and with picro-sirius red. Statistical and correlation calculations were performed as described by Sváb (1973).

Results

The results of the weekly weighings are presented in Table 3 and the weekly specific and cumulative feed utilization data in Table 4.

Table 3

Average weekly body mass gain (g)

		Week 2	Week 3	Week 4	Week5	Week 6	Week 7
Al–0	n	24	24	24	24	24	24
(Control)	x	277.29	491.46	797.71	1,143.13	1,526.38	1,906.81
	s	38.74	58.13	78.83	117.17	163.85	206.53
	cv%	13.97	11.83	9.88	10.25	10.73	10.83
	sx	7.96	11.86	16.09	23.91	33.44	42.15
Al–200	n	24	24	24	24	23	23
	x	277.50	493.33	790.83	1,134.58	1,496.52	1,839.13
	s	39.26	68.95	119.67	154.36	197.04	237.77
	cv%	14.15	13.98	15.13	13.61	13.17	12.93
	sx	8.01	14.07	24.42	31.50	41.05	49.53
Al–500	n	24	24	24	24	24	24
	x	277.50	501.67	800.00	1,143.75	1,511.67	1,837.92
	s	39.26	56.70	83.09	116.46	144.75	177.05
	cv%	14.15	11.30	10.39	10.18	9.58	9.63
	sx	8.01	11.57	16.96	23.76	29.54	36.13
Al–1000	n	24	24	24	24	24	24
	x	277.92	487.08	754.17	1,098.75	1,470.83	1,818.33
	s	39.01	67.98	105.50	141.52	205.68	249.90
	cv%	14.04	13.96	13.99	12.88	13.98	13.74
	sx	7.96	13.87	21.53	28.88	41.98	51.00
Al–P–200	n	24	24	24	23	23	22
	x	277.92	508.75	802.50	1,164.78	1,546.96	1,907.73
	s	39.01	68.92	110.66	149.51	198.82	244.77
	cv%	14.04	13.55	13.80	12.84	12.85	12.83
	sx	7.96	14.07	22.58	31.15	41.42	52.19
Al–P–500	n	24	24	24	24	23	23
	x	278.33	515.00	808.75	1,167.08	1,522.61	1,876.09
	s	39.64	64.81	103.75	133.17	178.08	206.81
	cv%	14.24	12.58	12.84	11.41	11.70	11.02
	sx	8.09	13.22	21.19	27.18	37.10	43.09

Table 3 continued

		Week 2	Week 3	Week 4	Week5	Week 6	Week 7
Al–P–1000	n	24	24	24	24	24	24
	x	278.33	482.08	470.42	1,031.67	1,446.67	1,698.33
	s	39.64	68.33	109.68	263.28	201.85	214.81
	cv%	14.24	14.18	14.81	25.52	13.95	12.65
	sx	8.09	13.94	22.38	53.73	41.19	43.84
Al–P–3000	n	24	24	24	24	24	24
	x	389.50	485.40	583.00	892.50	1,055.00	1,285.50
	s	74.58	91.60	120.88	253.29	211.77	276.01
	cv%	19.15	20.16	20.73	28.38	20.07	21.47
	sx	16.68	20.49	27.04	56.66	47.38	61.75

Table 4

Weekly and cumulative specific feed utilization (kg)

Treatment	Week 3	Week 4	Week 5	Week 6	Week 7	Cumulative
Al–0 (Control)	1.88	1.93	2.13	2.34	2.13	2.10
Al–200	1.91	1.87	2.10	2.34	2.32	2.13
Al–500	1.76	1.89	2.08	2.31	2.34	2.11
Al–1000	1.80	1.99	2.02	2.26	2.29	2.10
Al–P–200	1.78	1.96	2.25	2.31	2.96	2.26
Al–P–500	1.77	2.37	2.06	2.50	2.33	2.14
Al–P–1000	1.80	2.08	2.40	2.01	2.80	2.21
Al–3000	4.00	4.44	3.35	2.37	2.43	3.05

The average and extreme values of the 8 mineral elements measured in nine different organs of the 40 chickens, together with the standard deviation and coefficient of variation (CV%) of the values were summarized in tables not presented here. The concentrations of five mineral elements in nine organs of chicken groups subjected to different Al load are presented in Table 5. The numbers mean average values expressed in mg per kg of dry matter. The Mo, Co, Ni, Sr, Na and K concentrations found in 4 organs (liver, brain, testicle, bone) of some birds per group are summarized in Table 6.

Table 5

Accumulation of Al, Zn, Mn, Ca and P in 9 organs of broiler chickens exposed to different aluminium load

Group	Organ	Elements (mg/kg)				
		Al	P	Ca	Zn	Mn
Al–0	Liver	0.31	12.100	137.7	100.27	12.30
	Kidney	0.07	12.170	309.0	84.24	10.12
	Skeletal muscle	0.36	8.290	158.7	67.60	0.59
	Myocardium	0.17	8.958	200.5	114.13	2.55
	Brain	1.67	12.967	1,517.0	47.57	2.34
	Spleen	4.84	17.775	189.5	108.20	1.94
	Testicle		11.410	322.0	92.70	2.64
	Lungs	0.24	9.819	441.6	56.42	1.26
	Bone	22.87	155.160	318,270.0	361.60	21.53
Al–200	Liver	1.00	13.000	167.8	129.60	14.48
	Kidney	0.57	12.280	317.0	87.38	10.23
	Skeletal muscle	0.29	7.676	165.6	77.14	0.71
	Myocardium	0.93	10.272	199.6	113.90	2.75
	Brain	0.60	13.300	560.0	60.90	2.20
	Spleen		16.500	242.3	94.37	1.37
	Testicle	3.64	13.400	416.0	114.10	3.55
	Lungs	0.66	9.718	462.4	54.86	1.40
	Bone	16.22	149.880	332,380.0	391.80	9.77

Table 5 continued

Group	Organ	Elements (mg/kg)				
		Al	P	Ca	Zn	Mn
Al+P–200	Liver	0.37	12.680	172.8	129.80	14.68
	Kidney	0.60	11.940	338.0	90.78	9.21
	Skeletal muscle	0.63	6.798	145.2	66.68	0.64
	Myocardium	6.21	9.816	202.0	103.38	2.29
	Brain	0.29	15.867	826.0	50.83	2.07
	Spleen	0.11	16.150	166.5	87.00	1.38
	Testicle	0.29	13.800	525.0	98.20	2.60
	Lungs	0.20	0.848	401.6	60.08	1.12
	Bone	9.16	144.900	314,820.0	346.81	10.82
Al–1000	Liver	0.85	12.780	148.8	109.74	14.94
	Kidney	0.92	11.340	323.0	86.74	10.92
	Skeletal muscle	0.30	6.832	231.4	63.66	0.71
	Myocardium	0.47	10.248	217.6	100.14	2.53
	Brain	0.18	15.733	1,951.0	50.43	2.31
	Spleen	0.86	16.850	223.0	94.15	1.69
	Testicle	0.34	14.800	727.0	104.90	3.55
	Lungs	1.43	10.240	445.4	63.66	1.44
	Bone	19.68	151.320	327,180.0	367.20	9.44

Table 5 continued

Group	Organ	Elements (mg/kg)				
		Al	P	Ca	Zn	Mn
Al+P–1000	Liver	0.77	11.700	157.8	146.40	14.40
	Kidney	1.17	11.460	308.4	102.42	10.37
	Skeletal muscle	1.49	8.854	160.6	69.68	0.60
	Myocardium	0.38	8.902	212.6	103.66	2.10
	Brain	0.58	15.567	789.0	49.70	2.35
	Spleen	0.70	16.400	171.5	91.20	1.19
	Testicle	1.03	12.900	381.0	89.40	2.91
	Lungs	0.63	9.826	413.6	64.88	1.07
	Bone	36.66	152.400	371,640.0	378.60	10.93
Al–3000	Liver	3.37	11.120	153.7	122.24	15.58
	Kidney	3.26	11.630	277.3	88.38	12.26
	Skeletal muscle	1.45	8.580	161.1	74.33	0.72
	Myocardium	0.72	9.767	193.6	103.85	2.52
	Brain	1.06	16.125	546.0	56.98	1.89
	Spleen	6.62	15.833	185.3	94.17	1.27
	Testicle	1.97	13.400	610.0	95.80	3.46
	Lungs	3.68	9.986	341.3	61.28	1.43
	Bone	55.97	112.500	263,910.0	319.14	9.78

Table 6

Concentrations of six elements in four organs of the different groups

Group	Organ	Elements (mg/kg)				Na %	K %
		Mo	Co	Ni	Sr		
Al–0	Liver	3.25	-	-	0.307	0.309	1.08
(Control)	Brain	-	-	-	0.717	0.584	1.70
	Testicle	-	-	-	0.285	0.621	1.91
	Bone	-	-	-	68.200	0.449	0.15
Al–200	Liver	3.04	-	-	0.167	0.254	1.15
	Brain	-	-	-	0.745	0.562	1.76
	Testicle	-	-	-	0.644	0.666	1.77
	Bone	-	-	13.8	59.900	0.458	0.17
Al+P–200	Liver	3.19	-	-	0.089	0.245	1.19
	Brain	-	-	-	0.627	0.585	1.80
	Testicle	-	-	-	1.285	0.692	1.88
	Bone	-	-	-	56.100	0.418	0.13
Al–1000	Liver	3.55	-	-	0.095	0.281	1.14
	Brain	-	-	-	0.399	0.529	1.67
	Testicle	-	-	-	2.530	0.663	2.06
	Bone	-	-	-	33.900	0.424	0.11

Table 6 continued

Group	Organ	Elements (mg/kg)				Na %	K %
		Mo	Co	Ni	Sr		
Al+P–1000	Liver	2.91	-	-	0.136	0.311	1.11
	Brain	-	-	-	0.450	0.538	1.67
	Testicle	-	-	-	0.655	0.555	1.82
	Bone	-	-	14.3	52.900	0.443	0.10
Al–3000	Liver	3.75	-	-	0.266	0.280	1.03
	Brain	-	-	-	0.336	0.550	1.76
	Testicle	-	-	-	1.670	0.642	1.78
	Bone	-	-	-	31.700	0.510	0.25

Table 7

Results of correlation calculations for the elements measured in the liver

	Zn	P	Fe	Mg	Mn	Cu	Ca
Zn	-						
P	0.203	-					
Fe	-0.026	-0.355*					
Mg	0.438**	0.560***	-0.211				
Mn	0.315*	0.080	0.314*	0.428**			
Cu	0.573***	0.375*	-0.006	0.437**	0.400**		
Ca	0.482**	0.355*	0.064	0.567***	0.337*	0.332*	
Al	0.130	-0.471**	0.423**	-0.141	0.225	-0.066	0.184

$^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$

Table 8

Results of correlation calculations for the elements measured in the femur

	Zn	P	Fe	Mg	Mn	Cu	Ca
Zn	-						
P	0.637***	-					
Fe	0.162	0.338*					
Mg	0.604***	0.935***	0.285				
Mn	-0.025	0.323*	0.574***	0.290			
Cu	0.202	0.269	0.300	0.413**	0.314*		
Ca	0.619***	0.844***	0.283	0.781***	0.052	0.272	
Al	0.037	-0.150	0.174	-0.176	0.159	-0.133	-0.026

*P < 0.05; **P < 0.01; ***P < 0.001

Of the results of correlation calculations performed for mineral concentrations found in the liver, kidney, skeletal and heart muscle, lungs and femur, the data found in the livers and bones are presented in Tables 7 and 8.

Discussion

Disregarding the four broiler chickens culled between day 34 and 48 of the experiment, the birds remained symptomless and showed good feathering throughout the experiment. All chickens except those fed 3,000 mg/kg Al developed well. The body mass data (Table 3) show that the average body mass of chickens fed a diet supplemented exclusively with Al decreased very moderately (by 67, 69 and 88 g, respectively) up to the end of the 7th week, proportionately to the Al concentration, as compared to the control group. The average body mass of chickens fed a diet supplemented with 200 mg Al and P per kg did not differ, or was slightly higher, than that of the controls. At the same time, chickens fed a diet containing 3,000 mg Al showed a very expressed (621.3 g, 32%; Table 3) and well-visible (Fig. 1) reduction of body mass and were slightly anaemic, though at the beginning of the trial their body weight had exceeded that of the controls. However, no deaths occurred in that group either. In agreement with data reported by other authors (Storer and Nelson, 1986; Lipstein and Hurwitz, 1981; Elliot and Edwards, 1991), these results demonstrate that the prolonged feeding of a diet supplemented with at least 3,000 mg/kg Al is required to produce a significant body mass reduction in broiler chickens. However, the observation of other researchers (Hussein et al., 1990 *a*), suggesting that the adverse effect exerted by Al can be completely prevented by supplementing the diet with identical amounts of P, could not be substantiated. Besides the body mass reduction, this is supported also by the finding that the amount of Al almost doubled in the bone ash of birds of group Al+P-1,000 receiving P in a concentration comparable to that fed to the control birds.

In the three groups exposed to Al load the amount of feed used up for one unit of body mass gain showed minor fluctuations but did not exceed that measured in the control group. In chickens fed a diet supplemented with Al+P, feed utilization per unit of body mass gain exceeded that of the controls by at most 83 g in the 7th week of the trial.

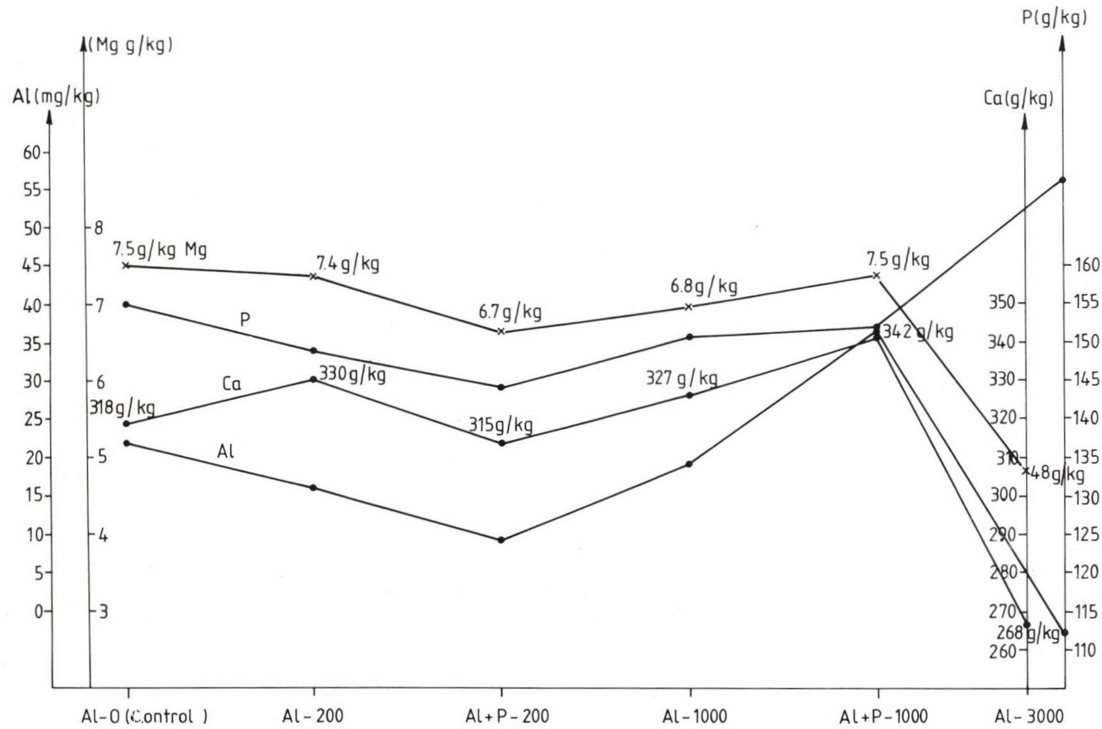

Fig. 1. Changes in the concentration of four elements (Mg, P, Ca and Al) as a result of the treatments applied

The only exception was the group fed 3,000 mg/kg Al, in which cumulative specific feed utilization reached 3.05 kg. This was approx. 30% higher than in the control group. These data are in contrast to those of other authors (Hussein et al., 1990*b*; Lipstein and Hurwitz, 1981) who reported a reduced feed intake, but are in agreement with the observations of Sooncharernying and Edwards (1990) who found impaired feed utilization. In agreement with the observations of other researchers, the very poor specific feed utilization of the group exposed to the heaviest Al load can be attributed to a transient acid–base imbalance caused by $AlCl_3$ fed without $NaHCO_3$.

As regards the group averages of elements measured and expressed for dry matter of the organs, the following statements could be made.

In the *livers*, the highest concentration of Al (average: 3.37 mg/kg) was measured in the 10 chickens fed 3,000 mg/kg Al. This concentration was about ten times that found in the controls. The Al content of the livers rose parallel to the increasing Al load. At the same time, in groups fed Al together with a similar amount of P the Al concentration of the liver hardly increased and was maximum twice as high as in the controls. This must have been due to the interaction playing a role in the absorption of the two elements.

Increasing doses of dietary Al tended to bring about a decrease in the Al concentration of the *bones*, which was below the control level even in chickens fed a diet supplemented with 1,000 mg/kg Al. The highest level (55.97 mg/kg), which was almost three times that of the control value, was found in chickens fed 3,000 mg/kg Al.

In the *other organs*, the Al concentrations showed a trend roughly similar to that seen in the livers and bones. Maximum Al concentrations in chickens subjected to the heaviest Al load were as follow: kidney: 3.26; skeletal muscle: 1.45; heart muscle: 0.72; brain: 1.06; spleen: 6.62; testicle: 1.97; lungs: 3.68 mg/kg.

At the end of the feeding trial the most pronounced Al accumulation was found, depending on the dose, in the livers, kidneys, lungs and skeletal muscles, while the highest and the lowest average Al concentration was measured in the bones (55.97 mg/kg) and in the myocardium (0.72 mg/kg), respectively. Aluminium accumulation was less expressed in the spleen, testicle and lung samples of groups fed Al in combination with a similar amount of P.

From the results it appears that while no major changes in Ca, P and Mg concentration occurred in the bones of chickens fed Al up to a dose of 1,000 mg/kg, the values of these elements underwent a very pronounced reduction (Fig. 1) in the group fed 3,000 mg/kg Al, in agreement with the observations of Elliot and Edwards (1991), accompanied by a marked simultaneous increase in the Al content of the bone ash.

Calcium concentration of the liver was higher in all the five experimental groups than in the control (137.7 mg/kg). The highest Ca concentration (172.8 mg/kg) could be measured in the liver of chickens fed a diet containing 200 mg Al+P. In the group fed 3,000 mg/kg Al, the Ca content was somewhat lower than in several other groups. The Ca content of the brain was the highest (1,951 mg/kg) in the group fed 1,000 mg/kg Al. The concentration of Ca in the testicles underwent a substantial, maximum almost twofold increase (to 727 mg/kg) in all Al-supplemented groups, whereas the Ca concentration of the kidneys, skeletal and heart muscle and lungs did not show a substantial and consistent rise even in the groups exposed to Al load.

The Ca content of the bones did not change substantially in the groups subjected to lower Al loads. As compared to the controls, the Ca content of the bones was slightly higher in chickens fed 1,000 mg Al+P and by approx. 20–25% lower (263,910 mg/kg) in the group designated Al-3,000.

Summing up the data presented in the tables, it can be established that the concentration of Al increased in the organs, first of all in the bones, of chickens fed 3,000 mg Al per kg of feed, while the concentration of Ca, P, Mn and, in all but one or two organs, Zn decreased. In respect of P and Zn, the results are consistent with the observations of other authors (Elliot and Edwards, 1991; Chung et al., 1990).

Cobalt could not be measured in any organ, whereas Ni was detectable, at a concentration of 13.8 and 14.3 mg/kg, only in the bones of the two groups of chickens fed Al (Table 6).

The average Mo content of the liver, kidney and heart muscle was 2.91–3.75, 2.49–3.14 and 0.17–0.92 mg/kg, respectively (Table 6). The Al load did not cause a substantial or consistent change in Mo concentration of the organs examined.

As a result of the Al load, the concentration of Sr decreased, though at a varying rate, in all organs except the testicle (e.g. in the brain it

dropped to half). At the same time, in chickens fed 1,000 mg/kg Al the concentration of Sr underwent a 2- to 8-fold rise, to a maximum of 2,530 mg/kg, as compared to the controls.

The Na content of the organs, calculated as group average, varied between 0.245 and 0.692% and hardly changed as a result of Al load. Average K content of the different organs was between 0.10 and 2.06%. The average K content of the bones increased from 0.15 to 0.25% as a result of the Al load.

The correlation calculations performed for 7 elements per organ revealed very close positive correlations between Mg and P, Mg and Ca and Zn and Cu in the liver (Table 7), Mg, Cu and P and Mg and Cu in the kidney, Zn and Cu, Fe, Mg and P as well as Cu, Mn and Fe in the skeletal muscles, Mg, Mn, Cu and P as well as P, Mg, Cu and Zn in the heart muscle, and between Al and Cu in the lungs. In the bones, there was a very close positive correlation between P, Ca and Mg as well as between Zn, Mg, Ca and P. A non-significant but negative correlation could be demonstrated between P, Mg, Cu, Ca and Al (Table 8).

At necropsy, the birds fed Al, primarily those fed a diet supplemented with 3,000 mg/kg Al, were less developed than the control chickens. In addition, in several of the chickens exposed to a heavier Al load the liver was a paler brownish-red and the gallbladder was markedly dilated. The lungs only showed lesions attributable to the exsanguination. The tubular bones showed no gross pathological lesions in any of the groups.

Histological examination of the experimental chickens revealed similar changes the severity of which increased with the rising dose of Al.

In chickens fed 1,000 mg/kg Al or 1,000 mg/kg Al plus 1,000 mg/kg P a mild focal oedema developed in the myocardium and in the brain. No pathological alterations were seen in the skeletal muscles, kidneys and spleen.

Cockerel chicks fed a diet supplemented with 1,000 mg/kg Al, 1,000 mg/kg Al plus 1,000 mg P, or 3,000 mg/kg Al showed proliferation of the biliary vessels, with moderate proliferation of the collagenic fibres of the surrounding connective tissue, vacuolar degeneration of the hepatocytes, solitary hepatocyte necrosis, and nuclear lesions (karyopycnosis, karyorrhexis, karyolysis) indicative of a toxic effect. By Gömöri's silver impregnation technique the reticular fibre network of the liver was found to be

intact (Fig. 2) in places where damaged hepatocytes (necrotic or degenerated liver cells, or those separated from the cell rows) were seen.

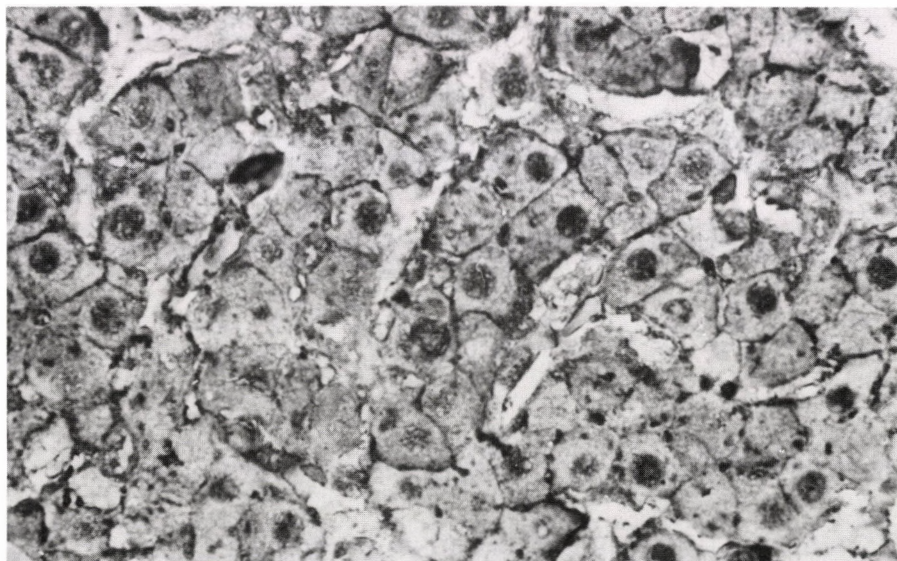

Fig. 2. Histological picture of the liver from a cockerel chick exposed to an Al load of 3,000 mg/kg of feed. In the area of solitary hepatocyte necrosis the reticular fibre network of the liver is intact. Gömöri's silver impregnation, × 1,050

Electron microscopy (EM) revealed partial degeneration and necrosis of the hepatocytes, dilatation of the granular endoplasmic reticulum, disintegration of the structure of, and the accumulation of electron-dense granules in, the mitochondria (Figs 3a and 3b). The myocardium showed diffuse interstitial oedema of moderate degree, and by electron microscopy myocardial fibre damage (fibrillar degeneration, disintegration of sarcomer structure) could be observed (Fig. 4). In the testicles, the interstitium was thickened, the amount of connective tissue elements increased and the number of Leydig's interstitial cells increased. Besides Sertoli's cells only few germinal epithelial cells were seen in the seminiferous tubules. Electron microscopic examination revealed partial degeneration in Leydig's cells and changes indicative of toxic effect (mitochondrial damage, fatty infiltration, and flattening of the saccules of the Golgi apparatus) in Sertoli's cells (Fig. 5).

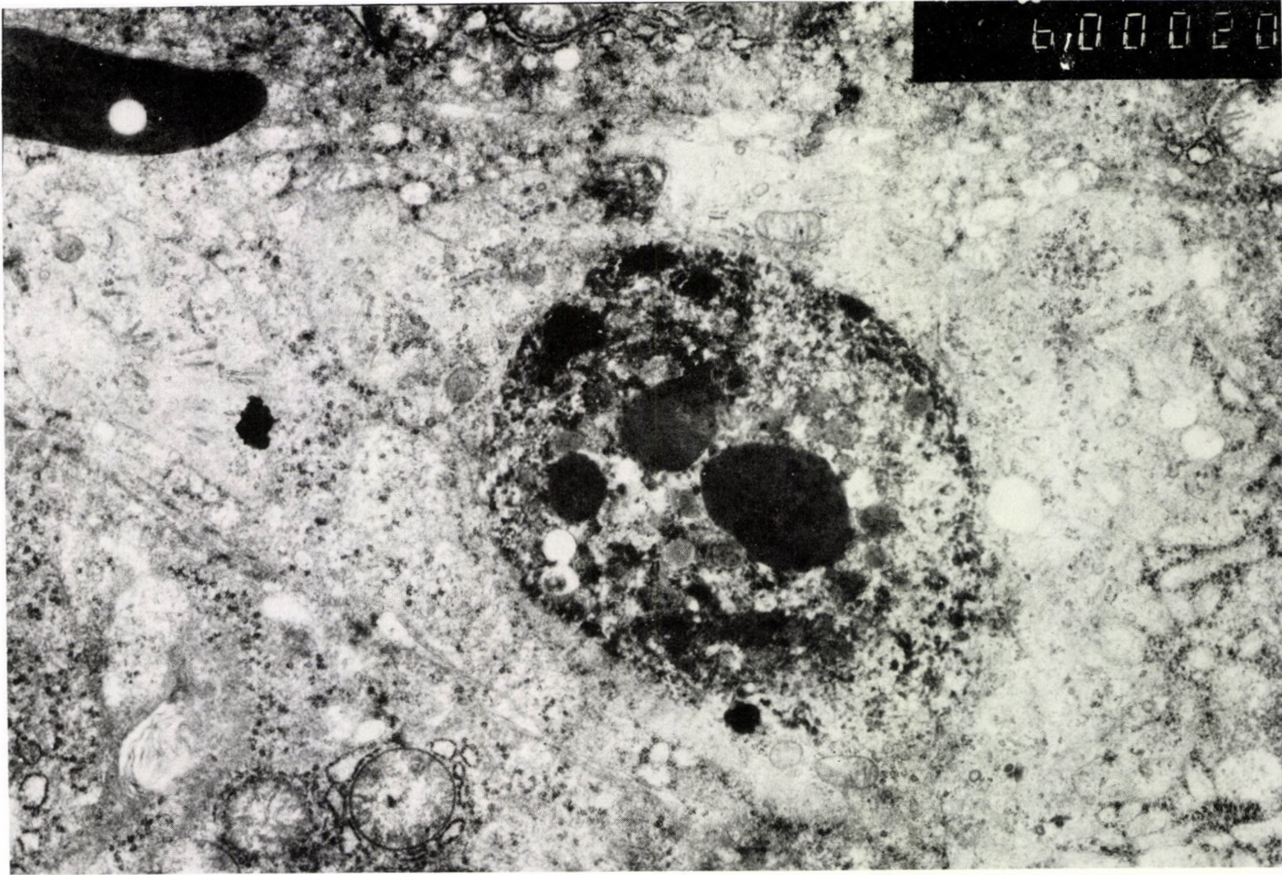

Fig. 3a. Electron micrograph of the liver from a cockerel chick exposed to an Al load of 3,000 mg Al per kg of feed. Note partial necrosis of the hepatocyte. × 13,200

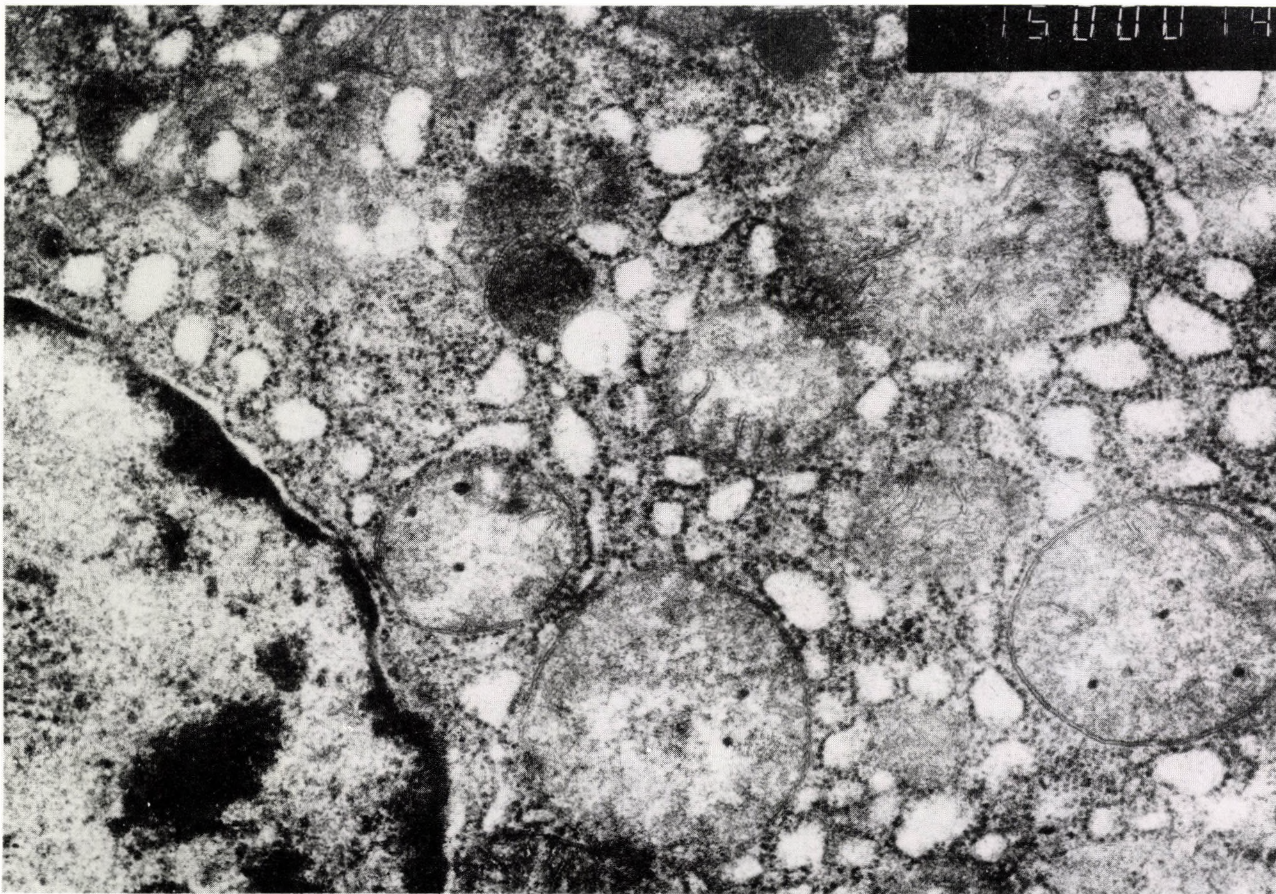

Fig. 3b. In the cytoplasm of the hepatocyte, note dilatation of the granular endoplasmic reticulum, disintegration of mitochondrial structure and the accumulation of electron-dense granules in the mitochondria. × 33,000

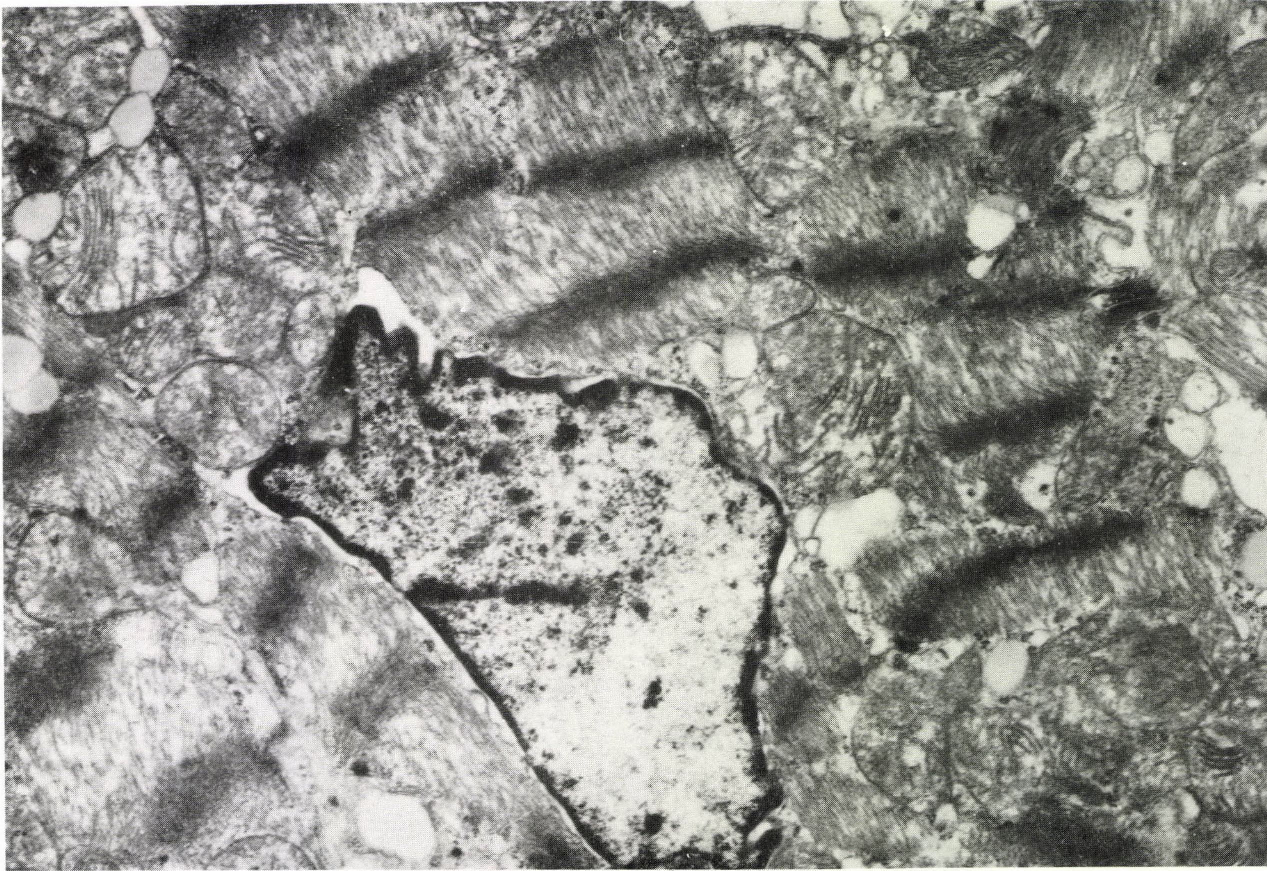

Fig. 4. Electron micrograph of the myocardium from a cockerel chick exposed to an Al load of 3,000 mg/kg of feed. Nore damage of the myofibrils and disintegration of sarcomer structure. × 37,500

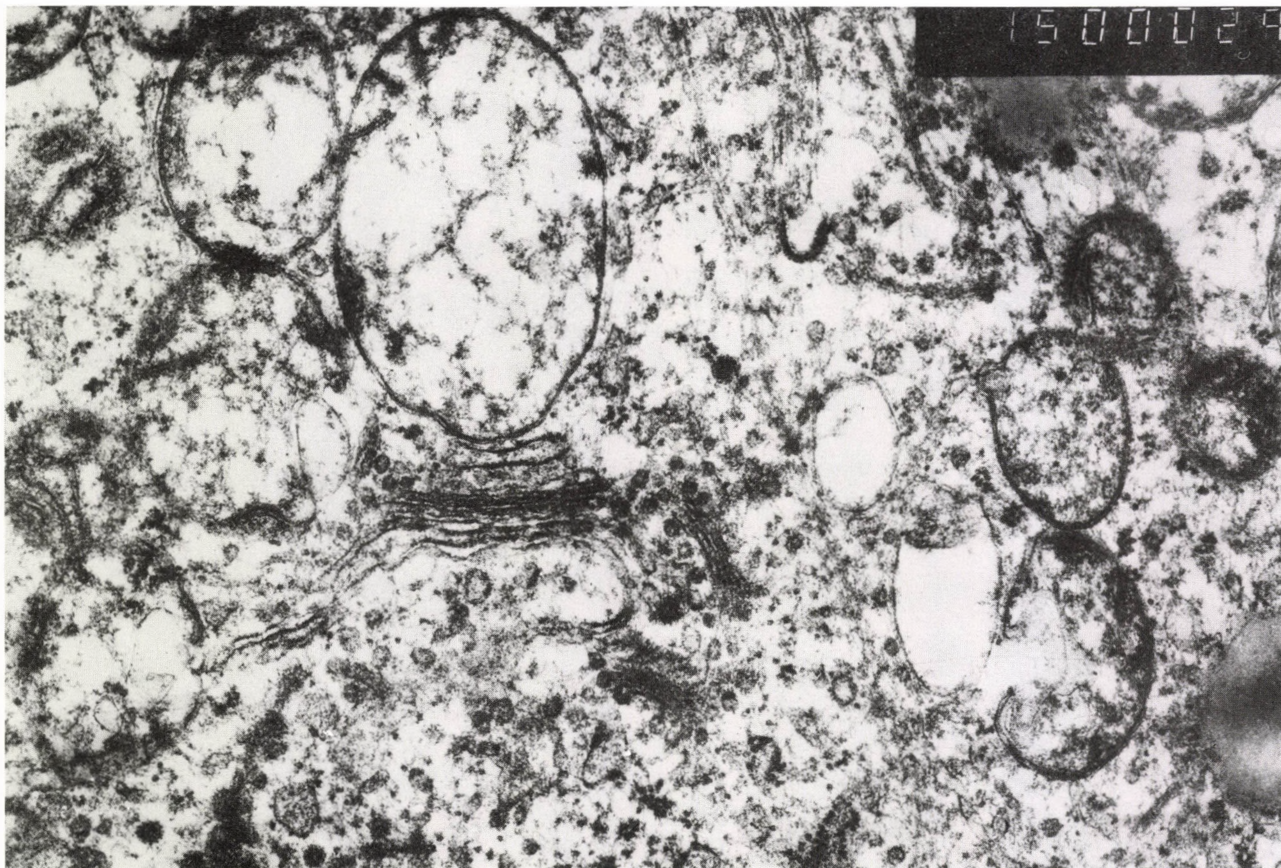

Fig. 5. Electron micrograph of the testicle from a cockerel chick exposed to an Al load of 3,000 mg/kg of feed. Note mitochondrial damage in the cytoplasm of Sertoli's cell and flattening of the saccules of the Golgi apparatus. × 13,200

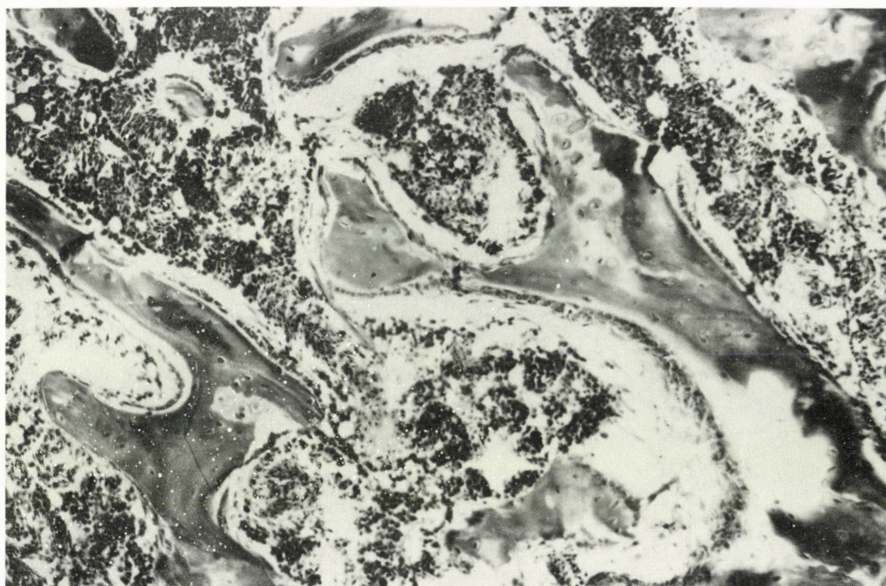

Fig. 6. Histological picture of the distal metaphysis of the femur from a control cockerel chick. Haematoxylin and eosin, × 160

Fig. 7. Histological picture of the distal metaphysis of the femur from a cockerel chick exposed to an Al load of 3,000 mg/kg of feed. Note flattened, inactive, partially degenerated osteoblasts on the surface of the thin bone trabeculae. Haematoxylin and eosin, × 160

By histological examination of the tubular bones, changes of similar character were seen in the experimental groups. The severity of these changes increased parallel to the level of Al supplementation. These changes were very mild and focal in two experimental groups which received P supplementation in addition to Al. As a result of Al supplementation of the diet, the following changes developed in the bones: the structure of the epiphyseal cartilage was normal. Supplementation of the diet with 200 mg/kg Al resulted in the focal proliferation of osteoblasts, the formation of non-calcifying osteoid islets and an increase in the number of osteoclasts. The cytoplasm of osteoblasts is slightly vacuolated. Higher Al doses (1,000 and 3,000 mg/kg of feed) produced, besides the above lesions, a large number of inactive osteoblasts in the metaphysis and diaphysis. These cells were flattened, had a narrow cytoplasm and a basophilic nucleus (Fig. 7). Many of them showed regressive changes (karyopycnosis, karyorrhexis). Osteoid synthesis was impaired in these areas. The compact substance of the bone was thin, the haversian canals and Volkmann's canals were dilated, and the number and size of bone trabeculae in the metaphysis was reduced as compared to the controls (Fig. 6).

In summary, it can be established that in the liver of chickens fed 1,000 and 3,000 mg Al per kg of feed the following changes developed: proliferation of the biliary vessels, moderate proliferation of the collagenic fibres, solitary hepatocyte necrosis, and nuclear lesions similar to those usually caused by toxic substances excreted in the bile. In addition, in chickens subjected to a heavy Al load (3,000 mg/kg of feed) partial degeneration of Leydig's interstitial cells and changes indicative of a toxic effect in Sertoli's cells were demonstrable in the testicles. In the metaphysis and diaphysis of the tubular bones of chickens exposed to a heavy Al load many inactive osteoblasts showing regressive nuclear lesions could be seen. The cerebral and myocardial oedema occurring in a severe form in the groups fed large doses of Al can be partly attributed to extermination.

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THE ROLE OF FEED PROTEIN QUALITY IN REDUCING ENVIRONMENTAL POLLUTION BY LOWERING NITROGEN EXCRETION
I. SURVEY OF PRINCIPLES IN PROTEIN EVALUATION FOR MONOGASTRICS: A REVIEW

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Environmental pollution by faecal nitrogen excretion motivates a renewed interest in optimising protein utilization of growing-finishing pigs in intensive pig production areas. The importance of digestibility of proteins, availability of amino acids, and the extent of protein retention is emphasized for reducing nitrogen excretion. The principles of various chemical, microbiological, biochemical and biological methods in feed protein quality evaluation offering a better adjustment of protein supply to the requirements of the animal are surveyed.

Key words: Feed protein quality, prediction of protein utilization, nitrogen excretion, principles of methods

Increasing environmental pollution motivates a renewed scientific interest in optimizing protein supply of farm animals to minimize faecal nitrogen emission. In intensive pig production areas pig manure and slurry are produced in large amounts and their nitrogen content is causing water (nitrates) and air (ammonia) pollution. Studies on possibilities to decrease the amount of nitrogen in animal effluents revealed that a better adjustment of nutrient supply in the feed (dietary protein and amino acid supply) would be needed.

Numerous factors related to animals or management may have an effect on nitrogen excretion of animals, however faecal nitrogen excretion depends primarily on dietary protein intake, protein digestibility, and on the extent of nitrogen retention, which depends primarily on the balance between the amino acids. To minimize nitrogen excretion, the importance of dietary protein quality and the practical utilization of the ideal protein concept were recently emphasized by several authors (Spiekers and Pfeffer,

1991; Gatel and Grosjean, 1992; Jongbloed and Lenis, 1992; Boisen, 1993)

This review gives a retrospective overview of the principles of methods which may be efficiently used in optimizing the protein and amino acid supply of monogastrics to achieve maximum weight gain and minimum nitrogen excretion.

Methods for determining the digestibility of proteins and amino acids

The digestibility (apparent or true) of the protein and the individual amino acids in the animal can be measured by balance methods. The *faecal digestibility* method developed originally by Kuiken and Lyman (1948) measures the difference between the amount of each of the amino acids consumed and excreted in the faeces. These apparent digestibility values may be corrected for the endogenous excretion of amino acids. This is usually determined by analyzing the amino acid levels in the faeces of animals which are fasting, or being fed a protein-free diet, to obtain true digestibility values.

The limitation of the faecal digestibility method is that endogenous amino acid losses are dependent on many factors (intake of dry matter and protein, level of crude fibre, possible presence of antinutritive factors etc.). Moreover, the amino acid composition of the faeces is largely unaffected by the amino acid pattern of the protein consumed (Bergner et al., 1980). The reason for this is the intense fermentation that occurs in the large intestine of rats and pigs. Thus approximately 60–70 per cent of the total nitrogen of the faeces is of bacterial origin.

The *ileal digestibility* of amino acids can be measured with surgically modified animals in which the intake and the amount of each amino acid passing through the distal part of the ileum are determined. The advantage of this method is that the confusing effect of the microflora in the large intestine can be avoided (Varnish and Carpenter, 1971; Zebrowska, 1978). However, the cannulation technique (simple vs. re-entrant cannula) and the method of sampling (spot vs. quantitative sampling) may affect the digestibility results (Drochner and Hazem, 1976; Hennig et al., 1980; Kubovics et al., 1989; Schröder et al., 1989).

The digestible amino acid content of the main feed ingredients has been extensively studied (e.g. Hermann, 1970) and utilized for diet formulation (Wünsche, 1977).

The advantages and limitations of the methods and the factors influencing amino acid digestibilities have been discussed by several authors (Eggum and Jakobsen, 1976; Eggum, 1977; Thanksley and Knabe, 1984; Sauer and Ozimek, 1986).

The true digestibility of amino acids is regarded as being synonymous with bioavailability (Sibbald, 1987).

The digestibility of proteins can be measured directly by putting feed samples in permeable nylon bags and placing them in the lumen of the duodenum via a cannula (Petry and Handlos, 1978; Sauer et al., 1983). The small sample size and the freedom from correction for endogenous nitrogen have recently focused attention to these techniques. (e.g. Taverner and Campbell, 1985).

The digestibility of proteins can be simulated or predicted by means of *in vitro* assays. For the determination of *in vitro* enzymatic digestibility, various proteases have been used, e.g. pepsin (AOAC, 1980*a*), pepsin and pancreatin (Akeson and Stahmann, 1964; Babinszky et al., 1990), pepsin, trypsin and erepsin (Kerese, 1977), papain, leucine-aminopeptidase and prolidase (Dvorak, 1968).

For rapid determination of protein digestibility by measuring pH drop or titrating acidity after 10 min of digestion several multienzyme assays have been developed (Hsu et al., 1977; Pedersen and Eggum, 1983).

The *in vitro* enzymatic hydrolysis of proteins leads to equilibrium, which is determined by the hydrolysis constant. The *in vitro* hydrolysis is always imperfect, because the liberated amino acids accumulate and will thus inhibit the progress of hydrolysis. In the course of the *in vivo* hydrolysis of a protein in the animal, however, the liberated amino acids will absorb continuously into the blood stream. Thus the degree of the hydrolysis can reach that maximal level which is allowed by the nature and structure of the protein.

By measuring the quantity of free amino acids liberated during enzymatic hydrolysis, information can be obtained on the absorbable (digestible) quantity of each amino acid.

Because of their simplicity, enzymatic *in vitro* methods are widely used, despite their limitations, to predict *in vivo* digestibility in the animal

and to check the declared protein quality of various commercially available protein feeds (e.g. meat-and-bone meals, feather meals, etc.)

Methods for determining or predicting protein retention

The potential nutritive value of dietary proteins is determined primarily by the proportion of their available amino acids. This potentiality will then be utilized at different efficiency rates during the processes of protein metabolism, depending on the many factors influencing protein utilization, which may be independent of the amino acid pattern of the dietary protein (Hegedűs, 1992).

When the energy content of a diet is not adequate, a part of the protein may be utilized in catabolic processes to gain energy and so the efficiency of the synthesis of new proteins will be lowered. The efficiency rate is also reduced by increasing the protein level in the feed. Factors independent of protein quality, e.g. a suboptimal supply of vitamins and minerals, the presence of antinutritive materials (e.g. trypsin inhibitors) or the proportion of other energy-supporting nutrients (carbohydrates, fats) may also have an effect on the utilization of proteins and faecal nitrogen excretion.

Because of these factors protein evaluation must be conducted under standardized conditions (e.g. at fixed protein level), where the rates of efficiency of utilization are the same. Data gained in this way can be used for ranking protein sources, monitoring changes in protein quality resulting from technological processing, or predicting the extent of nitrogen excretion.

In the course of practical feeding experiments protein quality may be examined under non-standardized circumstances as well, but conclusions based on such data can hardly be generalized.

The most practical way of evaluating feed proteins is to run feeding experiments. Unfortunately, these are rather time-consuming for getting preliminary information on a feed ingredient in time before buying or using it. Therefore faster and more reproducible *in vivo* and *in vitro* laboratory methods need to be used.

The quality of the protein may be characterized by various indices resulting from the method used. None of these indices can, however, be ex-

pected to reflect comprehensively the complex nutritive value of the protein. According to the character of the method, each index gives only a certain type of information about the quality of the feed protein. Therefore, an appropriate method must be selected with care regarding to the problem to be answered.

The expressions "quality", "biological value" and "nutritive value" of the protein are essentially synonyms. Nevertheless, the term "biological value" may be misleading as it can be easily confused with the "Biological Value Index" (BV) which specifically shows the percentage of the absorbed protein which is retained. Consequently, the term "protein quality" or "biological value" is more complex in its sense than the Biological Value Index.

The various methods which may be used for characterizing protein quality have been reviewed by several authors (e.g. Den Hartog and Pol, 1972; Pellett and Young, 1980; Sibbald, 1987; Heger and Frydrych, 1990), therefore only the principles of the methods are given here. This review may be of use to experimenters in choosing an appropriate method which enables the formulation of diets which allow the animal to excrete minimum quantity of nitrogen via the faeces and urine.

Chemical methods and scoring procedures

The procedure of complex evaluation of a protein source starts with the measurement of its nitrogen (crude protein) content, followed by the determination of amino acid composition of the protein. Nutritionists attempted to calculate various indices (scores) from the amino acid composition in order to predict *in vivo* protein utilization. These indices were in several cases modified by the overall digestibility of proteins and/or by amino acid availability.

The nutritive value of proteins depends primarily on their capacity to satisfy the needs of the animal for nitrogen (crude protein) and essential amino acids. This concept assumes a precise knowledge of these requirements. The basic principles of scoring procedures are to compare the amino acid composition of a protein with that of a reference pattern (requirement values), or to predict biological measures of protein quality

(e.g. *Biological Value*, BV; *Net Protein Utilization*, NPU) directly with the help of multiple regression equations.

The first scoring procedure has been introduced by Block and Mitchell (1946) and was called *Chemical Score* (CS). The values of each essential amino acid of a test protein are expressed individually in proportion to the content of a corresponding amino acid in a suitable reference amino acid pattern. The lowest proportion is regarded as limiting the protein utilization and is called "score". Scores and scoring patterns may be expressed in relation to protein or to nitrogen, on a percentage scale or as units per se.

The original concept of Block and Mitchell utilized the amino acid pattern of egg protein as a standard. Since then several other reference amino acid patterns or amino acid requirement patterns of the target animals have been proposed to obtain better agreement with biological indices of protein quality.

From a practical point of view only the amounts of lysine, total sulphur amino acids, tryptophan and perhaps threonine may need to be considered when calculating the scores of ordinary feedstuffs, because these are found to be the most frequently limiting.

The amino acid scores can be calculated in combination with protein digestibility or with available amino acid contents when data are available, to achieve a better correlation with biological indices.

Numerous indices have been proposed to predict biological measures of protein quality, utilizing different mathematical models (e.g. Oser, 1951; Mitchell, 1954; Mørup and Olesen, 1976; Berschauer et al., 1980; Glem-Hansen and Eggum, 1983; Hidvégi and Békés, 1985).

The concept of scoring procedures assumes that the essential amino acids have the same nutritional specificity, i.e. equal degrees of deficiency of any essential amino acid determine the same nutritive value of proteins. The available evidence, however, does not support this assumption. The differences in rates of degradation or conservation of essential amino acids in the amino acid pool result in differences with respect to the effects of a single essential amino acid deprivation on nitrogen balance and growth (Kino and Okumura, 1986; Dodds et al., 1987).

The limited accuracy of amino acid determinations, the uncertainty of amino acid availability, the different rates of amino acids released during digestion, the possible different utilization of free amino acids and proteins,

the role of non-specific nitrogen in amino acid requirements and protein utilization, the role of toxic or antinutritive materials affecting protein utilization independently of their amino acid composition have been extensively discussed as drawbacks of the scoring procedures. However, the advantages of speed, simplicity and low cost, as well as the possibility to identify the limiting amino acid and to estime the complementary value of a protein are considerable. Thus scoring procedures can be regarded as a valuable practical approach in optimizing amino acid supply and protein utilization to minimize nitrogen excretion via the faeces.

Microbiological methods

In comparison with enzymatic methods, microbiological tests relate better to biological methods. Microbiological methods of protein evaluation have been used for different purposes, as follows: (*i*) quantitative determination of essential amino acids from protein hydrolysates (Shockman, (1963); (*ii*) determination of relative protein quality, or the relative nutritive value of proteins (Hegedűs and Zachariev, 1978); (*iii*) determination of the availability of individual essential amino acids (Shepherd et al., 1977).

To compare the relative growth-promoting effects of protein hydrolysates, originally microorganisms without proteolytic activity (*Leuconostoc mesenteroides, Streptococcus faecalis*) were used. Later on the *Relative Nutritive Value* (RNV) of intact (or enzymatically pretreated) proteins was assayed by test organisms having proteolytic activity (*Streptococcus zymogenes, Tetrahymena pyriformis*). *Leuconostoc mesenteroides* was originally used by Horn et al. (1952) for monitoring heat processing damage of cottonseed. *Streptococcus faecalis* was successfully applied for measuring RNV values of various protein sources by Halevy and Grossowitz (1953). The method, based on the growth of *Streptococcus zymogenes* (Ford, 1964) was used for monitoring available amino acid content (especially methionine) of heat-processed protein concentrates, such as fish meal, meat meal and soybean proteins.

The essential amino acid requirements of *Tetrahymena pyriformis* W. have been claimed to be similar to those of growing rats (Holz, 1973). In adequate liquid media this ciliated protozoon propagates proportionally

to protein quality. Though strains of *Tetrahymena pyriformis* produce intra- and extracellular proteinases, enzymatic pretreatment of test proteins seems to be essential (Baum and Haenel, 1965). RNV values determined with Tetrahymena are sensitive indicators of protein damage or protein quality improvement. *Tetrahymena pyriformis* W. has been used as a suitable test organism for measuring availability of essential amino acids as well (Shorrock, 1976).

Protein Efficiency Ratio (PER) is a measure of protein quality which is based on the growth promoting capability of the protein. PER is used in the U.S.A. and Canada as an official method for regulatory (labelling) purposes. As *Tetrahymena* growth is claimed to highly correlate with PER values obtained in rats, the possibility of computing PER values from protein digestibility and *Tetrahymena* growth data was suggested (T-PER, Hsu et al., 1978).

The major weakness of microbiological assays of protein quality is the difference between animals and microbes in their ability to utilize peptides, as well as in their need for essential amino acids. Thus absolute values of microbiological assays must be treated with care when applied to higher animals. However microbiological assays are valuable tools to monitor relative changes in protein quality due to processing.

Biological methods

Biological methods for evaluating protein quality can be separated into several groups depending on the criteria used for assessing the response of the animal. The major subdivisions of the techniques are those depending on growth, nitrogen balance, and biochemical changes in organs or body fluids.

Some biological indices based on measuring changes in body weight are: PER = Protein Efficiency Ratio; NPR = Net Protein Ratio; NGI = Nitrogen Growth Index; GPV = Gross Protein Value.

Some biological indices based on the changes in nitrogen retention of the animal are: BV = Biological Value; NPU = Net Protein Utilization; NBI = Nitrogen Balance Index.

Methods based on changes in the body weight

The *Protein Efficiency Ratio* (PER) method measures the growth rate of young animals fed a test diet by relating body weight gain to the amount of protein consumed.

PER varies with the level of protein in the test diet, therefore the standardized AOAC (1980*b*) procedure recommends using 9.0 per cent crude protein. Because of its relative simplicity PER has been the most widely used animal assay in recent years. In the United States it is accepted in the legislation on protein foods for characterizing protein quality for labelling.

The PER method, however, has serious limitations. It shows merely the weight-promoting effect of the test diet, thus proteins of poorer quality, which do not promote weight gain (e.g. feather meals), cannot be tested with this method. The values of the PER index are not linearly proportional to protein levels in the feeds, therefore it is inappropriate as a quality estimate, where the product of protein quantity and protein quality is considered to express the amount of utilizable protein.

The PER is a single-dose assay, which does not include consideration of the effect of a protein-free diet, thus makes no allowance for proteins used for maintenance. Considering that a dietary protein with a NPU value of between 25–30 generally cannot be regarded as promoting weight gain, the zero PER value corresponds approximately to a value of 30 NPU. The PER method thus underestimates the feed proteins which are of poorer quality.

The *Net Protein Ratio* (NPR) method is an improvement over PER. It is defined as the weight gain of a test animal on a test diet plus the weight loss of a control animal on a protein free diet per gram of protein consumed by the test animal (Bender and Doell, 1957).

The values of NPR can be transformed to a scale of 0–100 by multiplying them by the mean nitrogen content of the animal body. The index thus obtained is named *Protein Retention Efficiency* (PRE).

The concept of NPR is comparable to that of NPU. It differs merely in that it is estimated from body-weight changes while NPU is calculated from body-nitrogen changes. Thus the correlation between NPR and NPU is usually high.

NPR values can be expressed also as percentages of a standard (e.g. lactalbumin or casein). This standardized assay is termed *Relative NPR* (RNPR).

The NPR method determines the response obtained using a single level of protein in the feed with reference to a protein-free control diet. The so-called "multi-level" or "multi-dose" assays are applying several levels of both the test and reference protein in the test feeds. In multi-dose assays the response of the animal (weight, body nitrogen, body-water) is related to the protein intake. Their concept is based on the supposition that the dose–response relationship is linear and the regression lines in case of different test proteins have a common intercept.

The *Relative Nutritive Value* (RNV-index, Hegsted et al., 1968) is based on the classic slope-ratio procedure using several levels of the test and reference protein. The dose–response relationship is then calculated by a multiple-regression model to ensure that all lines have a common intercept. This index was previously named "*Relative Growth Index*" (RGI) (Hegsted and Chang, 1965).

The *Nitrogen Growth Index* (NGI) of Allison (1955) differs from RNV (Hegsted et al., 1968) merely in the statistical method used for processing the data.

The *Relative Protein Value* (RPV) is the slope of the linear relationship between nitrogen intake (dose) and growth. The only difference from RNV is that the slope should not include the zero (protein-free diet) data. The response may also be expressed as body nitrogen or body water.

The *Gross Protein Value* (GPV) method measures the difference in body weights of a test group of animals fed a basal cereal diet together with the test proteins, and a control group fed the basal cereal diet alone. Values are calculated per gram of test protein consumed and referred to the value obtained using casein as a standard (Duckworth et al. 1961). GPV values show the supplementary values of protein to cereals and are in close correlation with the available lysine content of the protein.

The *Total Protein Efficiency* method (TPE) has been claimed to be an improvement of the GPV method (Woodham, 1968).

Methods based on measuring changes in the overall nitrogen balance

The classical *Biological Value* (BV) method determines the quantity of absorbed protein retained and is expressed as a percentage. If the BV value is multiplied by the digestibility of the protein, the NPU value can be obtained.

The *Net Protein Utilization* (NPU) method measures the quantity of the protein intake retained and is expressed as a percentage. It can be determined by nitrogen balance experiments (Bock et al., 1964) or by direct determination of carcass nitrogen content (Miller and Bender, 1955). Rats and chicks are both suitable test animals for the carcass method (Summers and Fischer, 1961). As the ratio of nitrogen to water in the animal proved to be constant the carcass method was later simplified by determination of body water content instead of nitrogen.

If the nitrogen-balance method is used, nitrogen intake and faecal as well as urinary nitrogen excretion are determined on the animal when fed diets both with or without protein. It is than possible to estimate not only net protein utilization but also apparent or true digestibility and biological value.

The percentage of the nitrogen retained from both the ingested or absorbed quantity of protein (i.e. the efficiency of the utilization of the protein) falls with increasing protein concentration in the diet. Therefore, it has been proposed that two terms should be used: NPU-standardized (NPU_{St}) at 10 per cent protein level in the diet, and NPU-operative (NPU_{0p}) at protein levels other then 10 per cent in the diet.

The *Nitrogen Balance Index* (NBI) method gives results essentially similar to the BV method, however it is based on a multi-dose assay. The nitrogen absorbed (dose) is plotted against the nitrogen balance (response) of the animal and then the slope of the line is measured (Allison, 1955).

The relationship of nitrogen retention to the nitrogen consumed is expressed by various terms in the German literature. These do not conflict, however, with those used in the English literature. The "*Netto Nutzwert*" used by Schiller (1957) corresponds to the NPU Index and the term "*Produktiver Eiweisswert*" used by Müller and Hötzel (1957) is the equivalent of the apparent NPU Index - as endogenous nitrogen losses are disregarded. The term "*Physiologischer Nutzwert*" used by Lintzel and Rechenberger (1940) is also a synonym of the NPU Index.

PER, NPR, BV, NPU, RGI represent overall estimates of protein quality. The limiting amino acid and its relative quantity in the protein, as well as other amino acids which occur in minimum proportions or in surplus are not explored by these assays. Accordingly, these methods are not applicable for the prediction of the complementary potential of proteins, but are suitable for ranking proteins and indicating certain technological effects (e.g. heat damage).

Methods based on changes in the nitrogen metabolism of various organs

A significant interaction can be demonstrated between the dietary protein quality and the nitrogen metabolism of various organs. The effect of the dietary protein may be detected e.g. by the degree of repletion of different organs depleted of their protein reserves.

The sensitivity of each organ to dietary protein quality has proved to be different and the following sequence has been observed: blood > liver > muscle > kidney > brain (Allison, 1964). To exploit this, numerous methods have been proposed, the most wide spread of which are the serum protein repletion (Chow et al., 1948) and the liver protein relation (Williams and Elvehjem, 1949).

These methods may give information concerning the utilization of dietary protein for special physiological functions.

The *Liver Protein Utilization* (LPU) method determines the proportion of feed protein retained in the liver (Mokady et al., 1969). Its concept is similar to that of NPU method.

Determination of the free amino acid content of the blood plasma after feeding has also been proposed for evaluating feed proteins. The free amino acid content of blood taken from the vena portae after feeding may increase proportionally with protein quality but is influenced by many factors, e.g. protein intake, the proportion of other components of the feed, the time interval between protein intake and blood sampling. These assays are therefore difficult to standardize.

The data of McLaughlan (1963) have supported evidence that the correlation between free amino acid levels of blood plasma and NPU may be poor. Similarly, a variable relationship can be observed between indices

calculated on free amino acid levels of the plasma and other indices of protein quality (Bodwell, 1975). As the free amino acid concentration of the plasma at the early stage of digestion depends on the quantities of liberated and absorbed amino acids, this method may be applicable for indicating the lower available amino acid content of proteins resulting from heat damage (Erbersdobler, 1973).

Urea concentration of blood plasma or serum has been shown to be negatively correlated with protein quality. The urea level depends on the quantity and quality of the ingested protein and the time interval after feeding (Münchow and Bergner, 1967; Eggum, 1973). The method can be standardized if the value obtained from the test protein is expressed as a percentage of its value obtained from a reference protein.

The activity of different tissue enzymes may depend on the quality of dietary proteins, thus theoretically their determination provides methods for protein evaluation. Extensive studies have been made on the activity of serum enzymes, liver arginase and xanthine oxidase as well as kidney transamidinase. Experience shows that these methods must be carefully standardized because the activity of the enzymes assayed depends not only on protein quality but on numerous other factors as well (Mauron, 1973; Bergner, 1974; Bodwell, 1975).

Conclusions

The concept of reducing nitrogen excretion of farm animals by improving protein quality of feeds implies that highly digestible ingredients must be used to allow maximum absorbtion of the nutrients. To achieve optimum retention of the absorbed amino acids the ideal proportion (pattern) of individual amino acids must be taken into consideration. The weight gain of animals is increasing with increasing dietary protein level between about 10–25 per cent crude protein in the feed; however, efficiency of protein retention decreases with increasing protein intake. Thus a proper balance must be found between weight gain and nitrogen excretion.

To optimize protein and amino acid nutrition of animals with a concomitant minimization of nitrogen excretion, various methods can be used to monitor or predict protein utilization. The appropriate method must be chosen with care and knowledge to achieve success.

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THE ROLE OF FEED PROTEIN QUALITY IN REDUCING ENVIRONMENTAL POLLUTION BY LOWERING NITROGEN EXCRETION
II. CHOICE OF AN ASSAY FOR MONITORING PROTEIN QUALITY AND PREDICTING PROTEIN UTILIZATION

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The optimum protein nutrition of farm animals allowing minimum nitrogen excretion is a compromise between performance (weight gain) and efficiency of protein utilization. The use of highly digestible feed ingredients allowing maximum nitrogen retention requires assay techniques which are suitable for monitoring protein quality of the feed or predicting nitrogen utilization of the animal. The various assay methods reflecting the protein metabolism of the animal may give different information according to the criteria, which they utilize. Therefore, a suitable assay should be selected with care, depending on the question to be answered. The various aspects of selecting assays for monitoring the protein quality of feeds and predicting protein utilization by the animals are discussed.

Key words: Protein quality, protein utilization, selection of assays, reduction of nitrogen excretion

The nitrogen excretion of farm animals can be reduced by feeding good quality proteins which are highly digestible and contain a well-balanced amino acid composition, which allows maximum nitrogen retention (Kienzle, 1992; Nieß, 1992; Wecke and Liebert, 1992). A precise adjustment of high-quality dietary protein and amino acid supply to the requirements of the animal by improving dietary amino acid balance using synthetic amino acids with a concomitant reduction of the dietary protein level may be the main strategies to reduce environmental pollution by nitrogen excretion (Eggum, 1980; Eggum et al., 1985; Kralovánszky et al., 1988; Goihl, 1990; Marbery, 1992).

The principles of methods suitable for monitoring protein quality of the feed ingredients and predicting protein utilization of the animal have

been reviewed in a previous publication (Hegedűs, 1992). This article discusses viewpoints to promote an appropriate choice from the various assay procedures.

The protein quality of a feed ingredient may be termed as the capacity of its protein content to fulfil the available amino acid requirement of the target animal. Considering the complexity of protein metabolism, the endeavour to characterize protein quality with a single index figure obtained by a selected method seems to be somewhat illogical. To judge dietary protein quality only a data sheet can furnish the necessary practical information such as:

- crude protein content;
- total amino acid pattern;
- limiting essential amino acid;
- *in vitro* digestibility obtained with proteinases;
- apparent or true digestibility obtained with the target animal;
- efficiency of protein utilization (BV, NPU, PER etc.);
- possible components with adverse effects;
- remarks (e.g. palatability).

Some criteria for the selection of an assay procedure are summarized in Fig. 1 and Tables 1–2.

Some remarks on planning animal assays

Sound planning and design are important to all bioassays. The objectives of the assay, species, genotype, sex, age of the test animal, the extent of the available facility and the analytical resources must be carefully considered in planning an assay. The samples may be ground or pelleted, dry or wet, and may be administered alone, or in complete diets as a sole protein source or as part of a mixture of other protein sources.

If the assay involves *ad libitum* feeding, the low palatability (e.g. purified diets) may limit feed intake. Pelleting may be used to improve palatability; however, ideally, test materials should be assayed in a form similar to that used under practical conditions.

As far as possible, test diets must be formulated to be isoenergetic, isonitrogenous and isofibrous, as the energy and fibre levels of the diets appear to cause major variations in protein utilization (Hegedűs, 1992).

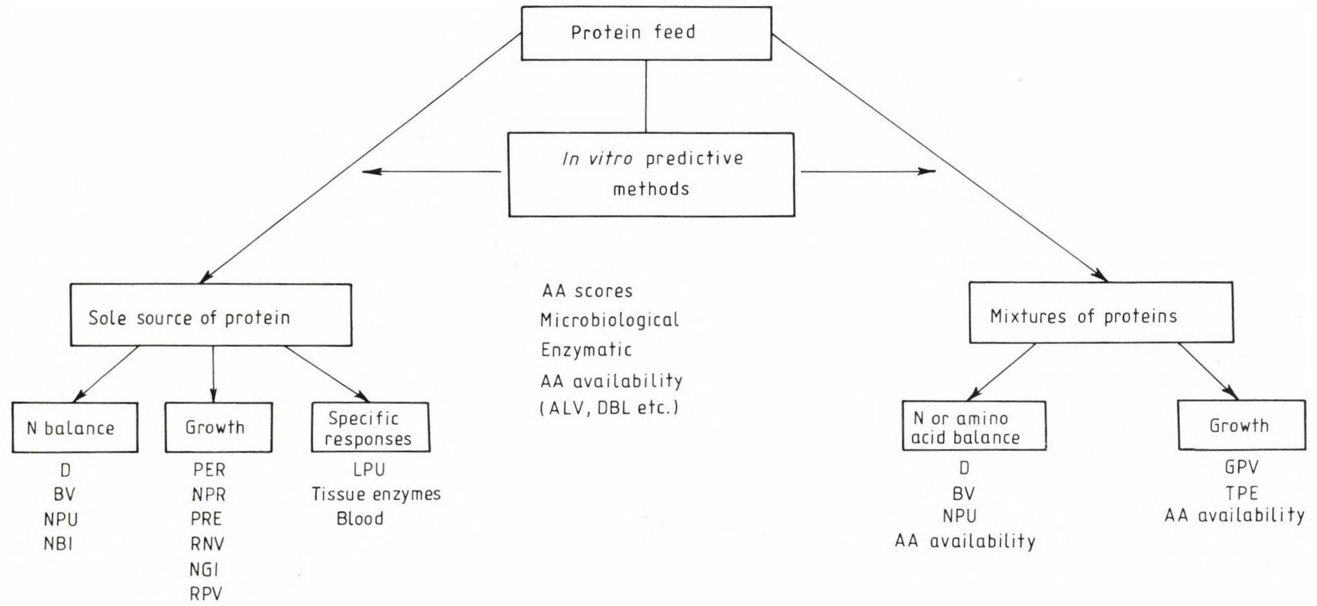

Fig. 1. A scheme for selection of an assay procedure

Table 1

Some criteria for the selection of an assay procedure

Type of information	Amino acid scores	Microbiological methods	*In vitro* enzymatic methods	*Amino acid availability*	
				Growth methods	Balance methods
Information for ranking protein	yes	yes	yes	no	no
Information for limiting amino acid complementary potential	yes	no	no	yes	yes
Information for growth promoting effect	no	yes	no	yes	no
Information for digestibility value	no	no	yes	no	no
Information for fulfilling amino acid requirements of farm animals for production	moderate	no	no	yes	yes
Suitability for screening	yes	yes	yes	no	no
Sample size required	small	small	small	moderate	moderate
Time required (days)	2	4	2	14	9
Relative cost	low	low	low	moderate	moderate
Simplicity of technique	simple	simple	simple	moderate	complex

Table 2

Some criteria for the selection of an assay procedure

Type of information	PER	NPR	NPU (carcass)	NPU (balance)	NGI	GPV
Information for ranking protein	poor	moderate	moderate	moderate	yes	poor
Information for limiting amino acid complementary potential	no	no	no	no	no	yes
Information for growth promoting effect	yes	yes	yes	no	yes	yes
Information for digestibility value	no	no	no	yes	no	no
Information for fulfilling amino acid requirements of farm animals for production	no	no	no	no	no	yes
Suitability for screening	no	moderate	moderate	no	no	no
Sample size required	moderate	moderate	moderate	moderate	large	large
Time required (days)	28	10–14	10	9	14	14
Relative cost	moderate	moderate	moderate	moderate	high	moderate
Simplicity of technique	simple	simple	moderate	complex	complex	moderate

The level of fibre and starch may affect microbial activity in the hindgut, thus influencing apparent protein digestibility values. If the test material is fed in a coarsely ground form, chicks tend to sort it, and the results of the assay may became fallacious. Sex, age and genotype of the animal have only a limited effect on protein utilization. Comparative trials are usually not consistent in this respect.

Cannulation, colostomy and caecectomy of chickens offer little advantage in digestibility trials, as the gut microflora has little effect on amino acid availability. In pigs, however, the effect of microflora in the large intestine is more pronounced, therefore surgically modified pigs are often used, making it possible to sample digesta before they enter the hindgut (determination of ileal digestibility).

Various techniques for caecum fistulation, caecectomy, ileocaecal cannulation, post-valvular ileocolic fistulation and ileo-rectal anastomosis have been developed as reviewed by Sibbald (1987). To arrive at a steady state of digesta flow, four-day collection periods are commonly used in nitrogen balance assays for pigs, poultry and rats alike. The preliminary acclimatization period of animals lasts from 2 to 7 days (most commonly 4 days), which must also include a frequent contact with the care personnel.

Ad libitum versus controlled feeding

Interpretation of the results of *ad libitum* feeding experiments is often complicated by the fact that the performance of the experimental animals is a consequence not only of the composition of the diet, but also of its amount consumed. Feed intake thus is a variable in growth responses.

Ad libitum feeding in many cases does not allow the separation of the metabolic efficiency response from the response to feed intake. However, the voluntary feed intake reflects on the palatability of the protein source, thus supplying valuable additional information about the acceptability of rations.

Controlled, equalized or restricted feeding usually results in a rapid consumption of the allotted diet by the hungry animal, thus the periods of fasting may alter the pattern of feed intake of nibbling animals (e.g. rats and chicks). In meal-eating species (e.g. pigs) restricted or controlled feeding may reduce the efficacy of synthetic supplements (e.g. lysine), because they

may be absorbed considerably faster than the natural nutrients. Restricted feeding of pigs in the practice results in leaner carcasses, which is often a demand of the market.

The old question, whether growth stimulation occurs because of feed consumption or whether intake stimulation occurs because of growth, is complex. However, weight gains are generally closely correlated with feed consumption. Growth response to a dietary protein probably also results from the effects of intake stimulation.

The effect of feed intake on the biological response of the experimental animal can be diminished if the nutrient to be tested is a limiting factor in the diet.

Controlled feeding for the same intake or for equal gains may be expected to increase the precision of nutrition experiments in comparison with the *ad libitum* variety. The feed consumption of laboratory animals may also be equalized by force-feeding techniques.

A compromise between *ad libitum* and restricted feeding is the so-called semi-*ad libitum* technique, where only a partial restriction of the feed intake is attained.

Scoring procedures: advantages and limitations

The role of feed proteins is to provide amino acids and nitrogen for the synthesis of tissue proteins and other nitrogenous metabolites. The knowledge of the amino acid content of feed proteins provides important information on limiting essential amino acids as well as on amino acids present in relative surplus.

Scoring procedures are simple, fast and cheap compared with bioassays. Calculations may be made also with values taken from feed composition tables. It is important however, that digestibility be considered.

The limitations of amino acid scoring (Chemical Score = CS; Essential Amino Acid Index =EAAI; Predicted Value = PV etc.) are that they do not reveal the extent of digestibility or availability of the protein or the amino acids, the relative surplus of amino acids and nonspecific nitrogen, the adverse effects of toxins or antinutritive factors. Moreover, these procedures cannot take into account the effect of the recycling of amino acids for protein synthesis, when they are present at suboptimal levels.

The problem of the accuracy of amino acid analyses and the imprecise reference amino acid pattern (the amino acid requirements of the target animal are not well defined) may also be mentioned as a potential drawback of these assays (Wang and Fuller, 1989; Fuller et al., 1989).

The relatively simple CS can acceptably predict biological estimates of protein quality (e.g. Net Protein Utilization = NPU) if the availability of the amino acids in the sample was not impaired. The more complicated indices calculated from amino acid composition are also insensitive to many of the factors affecting protein utilization, thus they often offer only little additional advantage over the chemical score.

Animal assays: advantages and limitations

The most commonly used animal assay procedures (Protein Efficiency Ratio = PER, NPU, Biological Value = BV) are dose dependent and often not well reproducible between different laboratories.

BV and NPU tend to overestimate the value of poor-quality dietary proteins low in lysine, and thus underestimate the needs of the animal from these proteins (Sammons and Hegsted, 1977).

Biological assays (PER, NPU, Net Protein Ratio = NPR, BV, etc.) are shown to be affected by various factors, such as high fat content, type of fat, carbohydrate level and source, high ash and NaCl level, crude fibre, biological antagonist (phytates, gossypol, avidin etc.), spices, flavours, non-protein nitrogen (nitrate, nitrite, urea etc.).

Biological assays are usually too complex for screening purposes. Their main advantage is their ability to show the overall usefulness of dietary proteins tested for meeting nutritional needs. However the amino acid composition, and consequently the complementary potential of the samples are not revealed by these assays.

Comparison of the balance technique with the carcass method in the assessment of nitrogen retention

The balance technique and the carcass method may give slightly different values for nitrogen retention. For instance in rats the nitrogen reten-

tion related to nitrogen intake (NPU) was 3.6 per cent higher by the balance technique than by the slaughter technique (Neergaard, 1981). In an experiment of Brüggemann and Niess (1984), the values for nitrogen retention obtained from the difference between N-intake and excretion (balance method) exceeded those from body analyses (carcass method) by up to 18 per cent.

The tendency to overestimate nitrogen retention by the balance technique can be explained by undetected losses of feed, faeces and urine, while the slaughter technique can lead to errors in the opposite direction. In the traditional balance method for the assessment of protein quality a sufficiently long preliminary period must be used to determine the amount of metabolic faecal nitrogen and endogenous urinary nitrogen for the BV values to become stable. Urinary nitrogen excretion, and consequently BV is often stabilized at a lower level of intake of lower quality protein only after a preliminary feeding period of 12–15 days (Eyre, 1984). The commonly employed preliminary period is, however, 5–6 days only.

Both the balance technique and the slaughter method are sufficiently accurate and reliable, however the slaughter technique has a slightly lower reproducibility. The balance technique is more complex and laborious than the slaughter technique, but it gives more detailed information (values for digestibility and endogenous nitrogen losses).

The use of l to 4 percent casein or egg protein in the control diet to avoid the assumed non-physiological conditions resulting from the protein-free diet has resulted in no additional advantage over the original carcass method (Lin and Huang, 1986).

The nitrogen concentration of rat carcasses within the circumstances of the conventional assay of BV is relatively constant, thus the nitrogen content of the body can be obtained by multiplying the weight of the carcass with its nitrogen concentration. The range of nitrogen concentration of dry carcasses is between about 8.1 and 8.7% (Miller et al., 1973). Because of the low variability of the nitrogen concentration of dry bodies and carcasses, the nitrogen content of the body, as well as the NPU values can be well predicted from growth data. Body nitrogen can also be well estimated from body weight, carcass water and carcass nitrogen by linear regression equations (Phillips, 1980).

In general, weight gain data from rats aged 21 to 60 days are as descriptive of the responses to experimental diets as the more laborious pro-

cedures requiring nitrogen analyses of the body or carcass (Rasmussen and McCully, 1972).

If the slaughter technique is used, the starting weight of the animals should be carefully equalized. Using the balance method, however, variation in the body weight and the age of the starting animals is of minor importance.

Choice of a nitrogen balance trial

Balance trials can be conducted measuring excreta either at the end of the ileum or at the end of the entire gut.

There are numerous comparisons between ileal and faecal digestion. Ileal digestibility of proteins or amino acids are found to be more sensitive to differences among feeding stuffs. However, the apparent ileal digestibility values tend to be 4–7 per cent lower than the corresponding faecal values. Ileal digestibility data may be affected by the unabsorbed endogenous faecal nitrogen, whereas faecal values are affected by the nitrogen originating from microbiological activity in the large intestine. To correct the nitrogen balance for gut microflora is of greater importance for pigs than for birds.

The superiority of "true" digestibility values over "apparent" digestibility data is subject to debate. "True" protein and amino acid digestibility values characterize more the quality of the feed protein *per se*, while "apparent" digestibility data present an overall response that integrates the gastrointestinal processes and the metabolism.

Methods applicable for regulatory purposes

At present official regulations requiring labelling to characterize the quality of proteins exist only in the U.S.A., where the PER values of certain human foods must be indicated. The limitations of PER, however, have been widely discussed by many authors. The main disadvantages being the poor reproducibility, the length of time required (five weeks) to produce data, and its sensitivity to many factors.

According to recent opinions, the NPR may be highly recommended for the purposes of monitoring the protein quality of foods.

Until recently there were no regulations regarding the labelling of the quality of the protein in industrial feed mixtures. These are commonly characterized by the *proximate chemical composition* (dry matter, crude protein and certain essential amino acids, crude ash, crude fibre, ether extract, minerals, vitamins, special additives), *energy* content (digestible, metabolizable or net values) and apparent *digestibility* coefficients of the main nutrients.

In the case of special protein feeds, where a statement is made about their high quality, the use of the NPR method may be recommended for indicating protein quality for possible regulatory or labelling purposes.

For screening or other purposes such as quality control, feed manufacturers may use whatever method they like. However, since many of the feed proteins are used for young growing animals, for regulatory purposes it seems appropriate to apply an assay that uses a growing animal (rat, chicken).

Practical evaluation of feed rations and mixtures

The classical assays for evaluating dietary proteins are regularly used to characterize sole protein sources under conditions where the efficiency of protein utilization is maximal (i.e. at low, about 10 per cent protein level). The ultimate aim of animal feeding is, however, to achieve maximum productivity at minimum cost, which can usually be realized by using higher dietary protein levels. Growing broiler chickens require, for instance, 23–16 per cent crude protein in the feed which results in a decrease in the efficiency of protein utilization and in a concomitant increase of nitrogen excretion.

The usual bioassays (NPU, BV) seem to be inappropriate in evaluating the capacity of feed proteins to supply available amino acids for meeting the quantitative requirements of farm animals under practical conditions of production.

The capacity of a feed ingredient to fulfil protein needs is determined by both the concentration and the quality of the protein since the amount and quality of the protein are considered to determine the quantity of

utilizable protein. However, this is an expectation, which is difficult to meet.

The standardized biological indices of protein quality (BV, NPU, etc.) seem not fully appropriate to express the utilizable (available) part of the protein by multiplying the crude protein content with these indices under practical feeding conditions, because they are always changing (decreasing) with the changing (increasing) protein level in the feed.

Protein requirement values for production are regularly expressed in terms of crude protein (poultry, pig) and apparent digestible crude protein (pigs). To express protein requirements in terms of available protein (crude protein content of feed multiplied by NPU values) seems to be impractical, because the actual protein retention of the animal is influenced by many factors.

To compare the capacity of the feed to fulfil the protein requirements of farm animals for production, the protein quality obviously must be expressed in terms identical with those of the requirements. Therefore in formulating rations for farm animals the crude protein, apparent digestible crude protein, total amino acid content and available amino acid content (the latter is practically identical with the true amino acid digestibility by faecal method) may deserve prime attention.

For the compounded feed industry the use of availability data of each amino acid is considered to be better than total amino acid data in formulating ratios by computers with least cost linear programming. Unfortunately amino acid availability data of feed ingredients are scarce and they are uncertain because of the variations depending on the composition and adequacy of the diet as a whole, and on the physiological, nutritional and health status of the animal. Therefore the total amino acid values are regularly used in diet formulations with the assumption of about 80 per cent digestibility as an overall safety level.

Biological indices of protein quality (BV, NPU etc.) may however be used in the practice for ranking conventional or unconventional feed ingredients and for monitoring changes in protein quality during processing or supplementation.

The current system of quality evaluation of sole protein sources does not recognize the fact that the nutritive values of low-quality proteins may be enhanced greatly when they are consumed together with other proteins.

To predict the protein quality of mixtures of two or more ingredients, the content of amino acids and an estimate of the nutritional availability of the limiting essential amino acid and that of the other essential and non-essential amino acids seem to be necessary.

Feed mixtures can exhibit complementary effects of different types (Bressani, 1977). Several amino acid indices were shown to predict the complementary effects of mixing ingredients (Woodham, 1978; Hegedűs et al., 1983*a*). For proof of possible synergistic effects, however, values predicted by *in vitro* indices must be born out by animal assays (Hegedűs et al., 1983*b*,*c*).

Correlation among protein quality assays

Dietary proteins may be assayed as sole protein sources or in mixtures with other protein-containing feed ingredients. The purpose of the experiment may be also different: to assess the *overall value* of a dietary protein to fulfil nutritional needs (PER, NPR, NPU, BV, Relative Growth Index = RGI, etc.) to assess the capacity of a dietary protein to *supplement* other protein sources (Gross Protein Value = GPV, Total Protein Efficiency = TPE, Dye Binding Lysine = DBL, Available Lysine Value = ALV, etc.), or to measure the *digestibility* and the *availability* of a protein or amino acids.

The appropriate method is to be selected carefully according to its principle and the question to be answered. Some relatively simple methods may be successfully used to predict more complex biological estimates of protein quality.

Protein utilization may be estimated by measuring nitrogen retention with animal assays (e.g.: BV, NPU, PER, NPR). Amino acid scores depending on the concept of limiting amino acids (CS, EAAI, etc.) may correlate well with these indices, particularly if the availability of the amino acids is not impaired.

If a newly developed method is supposed to refer to nitrogen utilization, the BV or NPU is commonly taken as the reference for comparisons. In cases of close correlation between two assays the lack of similarity in absolute values is of minor importance because of the possibility to interpolate on the regression line, if confidence limits are in a narrow range.

The correlation between two methods may also be negative. For instance the urea concentration of blood plasma is shown to correlate negatively with protein quality.

NPU or NPR methods may be recommended for comparisons as methods of reference in assessing nitrogen retention, where nitrogen retention values are related to intake nitrogen instead of absorbed nitrogen. Intake values are considered to be more practical from the point of view of animal feeding.

NPU and related methods usually give close correlation. Liver nitrogen retention (Liver Protein Utilization = LPU) may also be used instead of nitrogen retention of the whole body.

The weight and body nitrogen content of young, rapidly growing rats show a close correlation, which explains the close correlation between NPU and NPR values. If an appropriate calibration curve is available, NPU values may be obtained measuring body weights only and using interpolation on the graphs. The possibility of an unusual accumulation of fats or water in the rats fed test diets of unconventional origin must be excluded if using regression equations to predict NPU from NPR.

Microbiological assays with *Streptococcus zymogenes* or *Tetrahymena pyriformis* W. also have the potential to predict NPU. However, the degree of correlation and the extent of residual standard deviation (error of interpolation from the "x" data) do not allow too precise an estimation.

Digestibility estimates may not be correlated with protein utilization data. Digestibility refers to the amount of amino acids absorbed, whereas nitrogen retention (BV, NPU) estimates the amount of amino acids utilized. As has been discussed earlier, the utilization of absorbed amino acids is affected by various different factors (proportion of amino acids, amount of protein, dietary fibre content etc.).

The nitrogen content (raw protein content) of feeds is also an uncertain indicator of protein quality and in the vast majority of the cases has no informative value at all on protein utilization.

BV, NPU, NPR, and PER methods estimate protein utilization roughly according to the limiting essential amino acid. Thus an early impairment during heat processing of the availability of amino acids which are in surplus in a protein meal (e.g. lysine in blood meal) cannot be revealed by these methods, if they are not limiting.

To assess the availability of an amino acid which is in surplus in a supplementary protein, specific methods must be used. Methods estimating available lysine or methionine content must correlate well with animal assays.

GPV, ALV, DBL, DNFB-reactive lysine or microbiological methods for available lysine regularly show close correlation with each other or with animal assays.

The examples presented here try to illustrate that to solve specific problems methods may substitute for each other, according to the resources (facility, animal house, instrument, costs etc.) available in different laboratories.

Collaborative studies for comparisons of chemical, enzymatic, microbiological and biological assays for the estimation of protein quality and available amino acids have been published by several authors (e.g. Carpenter and Opstvedt, 1976; Njaa et al., 1980; Zombade et al., 1980; Nordheim and Coon, 1984; Porrini and Testolin, 1984; Batterham et al., 1984; Sarvar et al., 1984.).

Conclusions

The optimum protein nutrition of farm animals allowing acceptable performance with minimum nitrogen excretion is a compromise between net income and environmental pollution. Protein supply ensuring maximum growth inevitable results in a reduced efficiency of protein utilization and an increased nitrogen excretion, which in dense pig production areas may cause environmental pollution. The use of highly digestible feed ingredients with good protein quality which allow maximum nitrogen retention requires assay techniques suitable for monitoring protein quality or predicting nitrogen utilization. The various assay methods reflecting the protein metabolism of the animal may give various information according to the criteria which they utilize. Therefore a suitable assay, which helps to optimize protein nutrition of farm animals, should be selected with care, depending on the question to be answered.

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DIFFERENCES IN BIOCHEMICAL PROFILE FROM ACTIVE AND INACTIVE OVARIAN TISSUE IN RELATION TO OESTROUS CYCLE PHASES*

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The differences in biochemical components between active and inactive ovarian tissue were studied during the oestrous cycle. Goats (Marwari breed) were slaughtered at six different phases of the oestrous cycle (n=5) such as oestrus (day 1), ovulatory (day 2), early luteal (day 3), late luteal (day 9), follicular (day 15) phase, and goats having smooth ovaries without follicles or CL (SO). The levels of total protein, soluble protein, alkaline and acid phosphatase were significantly different ($P<0.05$) at different phases of oestrous cycle. Differences in nucleic acids (RNA and DNA), ascorbic acid, free cholesterol and esterified cholesterol were non-significant. The levels of proteins (total and soluble) and nucleic acids were high in the active ovary on day 9 (peak luteal phase) and day 15 (follicular phase) of the cycle. The activity of both alkaline and acid phosphatases was higher on day 2 (ovulatory phase) in the active ovary as compared with the inactive ovary. Highly significant ($P<0.01$) differences in total cholesterol concentration were found between different phases of the oestrous cycle. Maximum utilization of cholesterol was observed in the peak luteal phase (day 9) of the cycle.

Key words: Ovary, activity, biochemichal profile, oestrous cycle, goat

Biochemical estimates of the factors that are known or considered to influence ovarian activity were made from ovarian tissue to correlate and understand the status of the active and inactive ovary during different phases of the .oestrous cycle. The main physiological functions of protein include the maintenance of normal blood volume and normal water content of tissue and fluids (Roubieck and Ray, 1972). Optimum protein level is necessary for the development of endocrine and sexual organs (Hemick, 1977). Protein deficiency affects the libido, sexual maturity, gamete pro-

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duction, puberty and body growth (Meeacham et al., 1963). Low protein exerts its adverse effect on reproduction through the pituitary affecting the sexual glands (Marynard et al., 1979). Soluble protein represents hypertrophy of the componentsof ovarian tissue and the enzymatic activity of gonadal tissue like the ovary (Harper et al., 1979). The phosphatase enzymes, both alkaline and acid phosphatase, participate in a number of cellular processes, such as cellular transport, the synthesis and metabolism of various subtances of different classes ranging from phospholipids and RNA to carbohydrates. These enzyme activities in serum and tissue vary with age, sex, species, breed, health status and various physiological conditions of the animal (Dempsey and Wislocki, 1945). Cholesterol is the most widely distributed steroid precursor in blood as well as tissue. Cholesterol is high during steroidogenesis, where it acts as a precursor for the synthesis of steroid hormones of the gonads and adrenal cortex (Villae, 1961; Taylor et al., 1966; Drofman, 1969). The significance of free or esterified cholesterol in a tissue may be related to the structural characteristics of the membrane of a particular tissue (Bartely, 1970). Biochemically esterified cholesterol was found to be more liable to change than free cholesterol which was more or less constant. Increase in cellular population due to mitotic division results in increased DNA content, whereas increased RNA content may be indicative of accelerated protein synthesis of cells. Ascorbic acid plays an important role in endowing the organism with enhanced resistance (Hawk et al., 1954). Cholesterol and ascorbic acid levels rise hand in hand, especially so far as steroid-related functions are concerned (Zarrow et al., 1964).

The main objective of the present study was to reveal differences in biochemical components (proteins, phosphatase, cholesterol, nucleic acids and ascorbic acid) from ovarian tissue between active and inactive ovaries during different phases of the oestrous cycle.

Materials and methods

Animals. Thirty Marwari goats were used for this study. All animals were 2 to 2.5 years of age, apparently in good health and free from all diseases. After observing regular cyclicity, these animals were divided into six groups. Each group comprised five animals. The animals were slaughtered

in the following phases of the oestrous cycle: day 1 (D1, oestrous phase), day 2 (D2, ovulatory phase), day 3 (D3, early luteal phase), day 9 (D9, peak luteal phase), day 15 (D15, follicular phase), and SO (goats with smooth ovaries without any follicles or CL (non-active phase).

After slaughering, ovarian tissues from both the active and the inactive side were removed and cleaned of fat.

Biochemical analysis. For biochemical estimation 20% homogenates were made of ovarian tissue and filtered through Whatman filter paper no. 1. All extracts were stored at 4 °C until used for the analysis. The estimations were done by standard methods.

Total and soluble proteins. Total and soluble proteins were estimated using the method of Lowry et al. (1951). Aliquots (0.1 ml) of ovarian tissue hormogenate were used. Protein was expressed in mg/g tissue units.

Alkaline and acid phosphatase. Phosphatase enzymes were estimated by the method of King and Armstrong (1934). The substrate (disodium phenyl phosphate) was allowed to act undeur standard pH and temperature. After the reaction, phosphate gives phenol with 4-amino antipyrene (with the help of bicarbonate which works as activator), reacts with potassium ferricyanide and gives red colour. For alkaline and acid phosphatase, phosphatase and citrate buffer was used, respectively. The intensity of colour was correlated with phosphatase activity and was read at 510 nm in a Spectronic 20 colorimeter. Activity was measured in terms of KA Units/g tissue.

Cholesterol (total, free and esterified). Cholesterol was estimated by the method of Schoenhemer and Sperry (1934). The protein was precipitated from the tissue extract with a mixture of accetone and alcohol (1:1). Total cholesterol was measured on the basis of the red colour produced by the reaction of cholesterol in acetic acid with ferric chloride and H_2SO_4. Free cholesterol was precipitated with digitonin, the developed red colour was measured in a Spectronic 20 colorimeter at 570 nm. The value of esterified cholesterol was calculated as the difference between total and free cholesterol. The value was expressed as mg/100 g of tissue.

Nucleic acids (RNA and DNA). Nucleic acids were estimated from the ovarian tissue homogenates by the method designed by Schneider (1957). The acid-soluble compounds were removed by washing twice with 10% trichloroacetic acid (TCA). Then the sediments were washed with

95% ethyl alchol to remove lipids. The remaining residue was mixed with 5% TCA and distilled water and heated at 90 °C for 15 min to separate the RNA and DNA from the tissue proteins, which was left as insoluble residue. The colour reaction was developed by orcinol reagent, which gives green colour, and for DNA by diphenyl amine which develops a blue colour. Both DNA and RNA were expressed in mg/g tissue.

Ascorbic acid. Ascorbic acid was estimated in the tissue extract using the method of Mamoru et al. (1978). It was extracted by grinding biological materials in a mortar with 6% HPO_3, containing 2N acetic acid. The slurry was centrifuged for 20 min at 5,000 rpm. One ml of supernatant was pipetted into two tubes. After 5 min 0.05 ml of 0.02% 2,6-dinitrophenol indo-phenol dye and 0.05 ml of 1% BrO_3 was added to the sample tube, and 0.05 ml of H_2O to the control tube. After 1 h all the tubes were incubated for 3 h with 1 ml of 2.05 thiourea in 5% HPO_3 and 0.05 ml of DNPH (dinitrophenyl hydrazine) in $9NH_2SO_4$ at 60 °C. The reaction was stopped by chilling. The ozone formed was dissolved by the addition of 2.5 ml of 85% H_2SO_4. The sample tube was centrifuged at 5,000 g for 10 min to remove turbidity. The absorbance at 540 nm was read against blank, which was prepared in exactly the same way except for the omission of DNPH. The value was expressed in μg/g tissue units.

Statistical analyses. The data collected from the experiments were tabulated and mean ± SEM and significance were calculated. The following analyses were done by the statistical methods suggested by Snedecor and Cochran (1971). (a) Completely randomized block design (CRD) for analysis for variance between the treatments. (b) Paired t test for comparing characters of active and inactive ovaries. (c) Duncan's multiple range test applied for comparing various means.

Results

Proteins (total and soluble)

Total protein concentration ranged from 18.55 ± 3.09 to 106.92 ±11.24 mg/g tissue and 14.26 ± 1.16 to 106.26 ± 11.24 mg/g tissue for active and inactive ovarian tissue, respectively (Fig. 1a). The oestrous phase (D1) and ovulatory phase (D2) showed similar trends in total protein con-

tent of the active ovary. On the inactive side, D2 (ovulatory phase) and D9 (peak luteal phase) did not show much variation between each other. The estimated differences between different phases of the oestrous cycle were significant at $P<0.05$ for both active and inactive ovaries. A comparison of active and inactive ovaries by the paired *t* test indicated that the total protein content yielded significant differences for different days of the oestrous cycle, except for D9 when the values were highly significant ($P<0.01$).

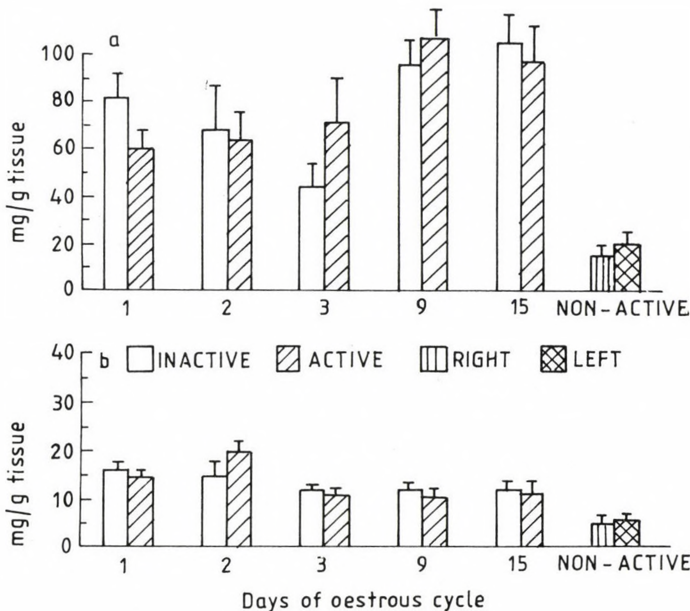

Fig. 1. Levels of (a) total protein and (b) soluble protein in ovarian tissue during different phases of the oestrous cycle. Values are mean ± SEM for 5 animals/group. ** $P<0.01$ (comparison between the two ovaries)

The average value of soluble protein was 11.87 ± 1.88 mg/g tissue for the active and 12.22 ± 1.77 mg/g tissue for the inactive ovary. The highest value was obtained for D2 (18.87 ± 1.6 mg/g tissue) in the active ovary but there were no differences in soluble protein concentration from D1 to D15 of the cycle in active ovarian tissue (Fig. 1b). Differences between D3 (early luteal phase) and D9 (peak luteal phase) were highly sig-

nificant ($P<0.01$) when soluble proteins of active and inactive ovaries were compared. Analysis of variance indicated significant ($P<0.05$) differences in soluble protein levels between different phases of the oestrous cycle, the levels were significantly different at $P<0.05$ for both ovaries.

The average value of acid phosphatase for active and inactive ovarian tissue was 0.60 ± 0.12 and 0.32 ± 0.05 KAU/g tissue, respectively. Analysis of variance of active ovarian tissue indicated significant ($P<0.05$) differences in acid phosphatase level between different phases of the oestrous cycle. The levels found in active and inactive ovaries were compared: the differences found in the peak luteal (D9) phase and the follicular (D15) phase were highly significant ($P<0.01$).

Cholesterol (total, free and esterified)

The levels of esterified cholesterol ranged from 445.71 ± 97.7 to 945.61 ± 55.81 mg/100g tissue for the active and from 565.71 ± 59.41 to 971.31 ± 50.11 mg/100g tissue for the inactive side (Fig. 2a). The average level of esterified cholesterol in the inactive ovary (701.16 ± 59.90 mg/100g tissue) was slightly lower than in the active ovarian tissue (707.83 ± 68.66 mg/100g tissue; Fig. 2a). Esterified cholesterol levels of the active and the inactive side were compared: no significant differences were found in any phase of the oestrous cycle. Statistical analysis revealed no significant differences in esterified cholesterol concentration between the active and the inactive ovary.

Mean total cholesterol level ranged from 485.71 ± 96.71 to 1151.42 ± 113.51 mg/100g tissue for the active ovary and from 771.42 ± 115.85 to 1171.42 ± 53.35 mg/100g tissue for the inactive side. The minimum value (485.71 ± 96.71 mg/100g tissue) was obtained in the follicular phase (D15) in the active ovary (Fig. 2b). There was no appreciable fluctuation in total cholesterol among oestrous cycle stages in the inactive ovaries. In active ovarian tissue there were highly significant ($P<0.01$) differences in total cholesterol levels between different phases of the oestrous cycle, but such differences did not exist in the inactive ovary. A comparison of active and inactive ovaries for total cholesterol level showed significant ($P<0.05$) differences only on D15.

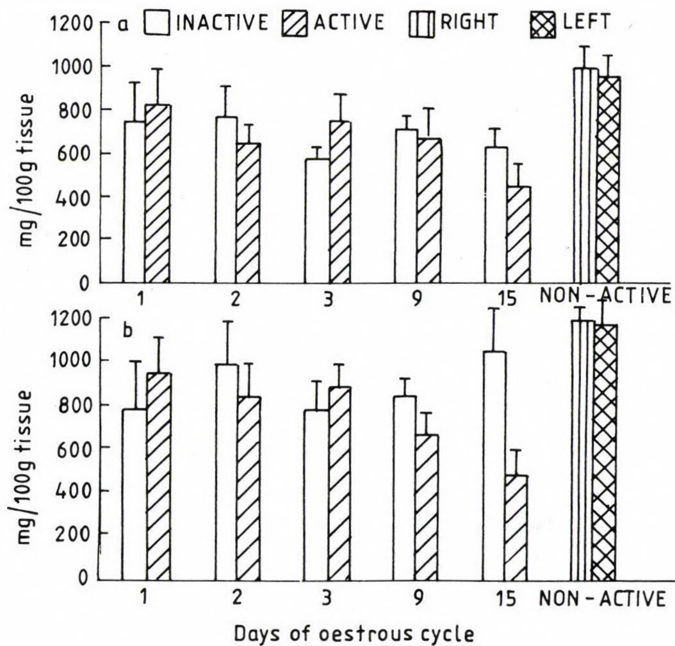

Fig. 2. Levels of (a) esterified cholesterol and (b) total cholesterol in ovarian tissue during different phases of the oestrous cycle. Values are mean ± SEM for 5 animals/group. * $P<0.05$ (comparison between the two ovaries)

The average level of free cholesterol was 146.65 ± 21.44 mg/100g tissue for the active side (extreme values: 79.0 ± 13.97 – 205.71 ± 55.81 mg/100g tissue) and 205.60 ± 55.81 mg/100g tissue for the inactive side (extreme values: 113.76 ± 43.70 – 411.31 ± 188.56 mg/100g tissue; Fig. 3). There were no significant differences in free cholesterol between the different phases of oestrous cycle on either side. With the exception of the follicular phase (D15) and the non-active (SO) stage, free cholesterol level did not show much variation on the active side, but on the inactive side free cholesterol found on D9 (peak luteal phase) and in the SO (non-active) phase were different. A comparison between active and inactive ovarian tissue showed that free cholesterol levels were significantly different in all phases except D15.

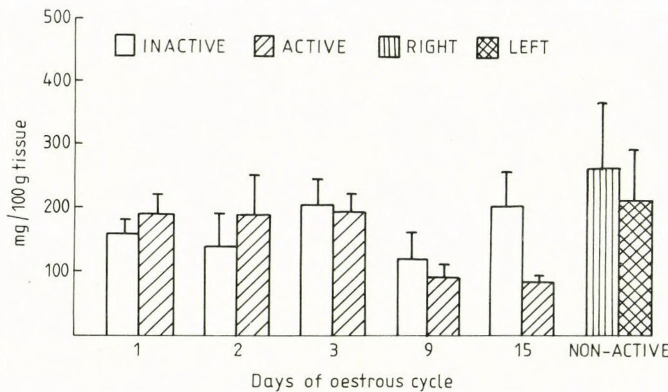

Fig. 3. Levels of free cholesterol in ovarian tissue during different phases of the oestrous cycle. Values are mean ± SEM for 5 animals/group. * P<0.05 (comparison between the two ovaries)

Nucleic acids (RNA and DNA)

The highest RNA level (0.35 ± 0.09 mg/g tissue) was present during the peak luteal phase (D9) and the lowest during the oestrous phase (D1) on the active side. The inactive side had the highest level (0.27 ± 0.03 mg/g tissue) in the early luteal (D3) phase and the lowest in the oestrous phase (D1). For the non-active (SO) ovary this value was 0.16 ± 0.11 mg/g tissue (Fig. 4a). Comparison of RNA levels between active and inactive ovarian tissue showed no significant differences on all days but D1. Between different phases of the oestrous cycle, differences in RNA levels were non-significant for both ovaries. Mean DNA concentration ranged from 2.47 ± 0.19 to 4.03 ± 0.81 mg/g tissue for the active side and from 2.15 ± 0.27 to 3.36 ± 0.57 mg/g tissue for the inactive side (Fig. 4b). Differences in DNA levels during different phases of the oestrous cycle were non-significant for both types of ovarian tissue. Maximum DNA levels (4.03 ± 0.81 mg/g tissue) were obtained on D15 on the active side and (3.36 ± 0.58 mg/g tissue) on D2 on the inactive side. When DNA levels of the active and inactive ovaries were compared, significant differences (P<0.05) were found on D2 and D3.

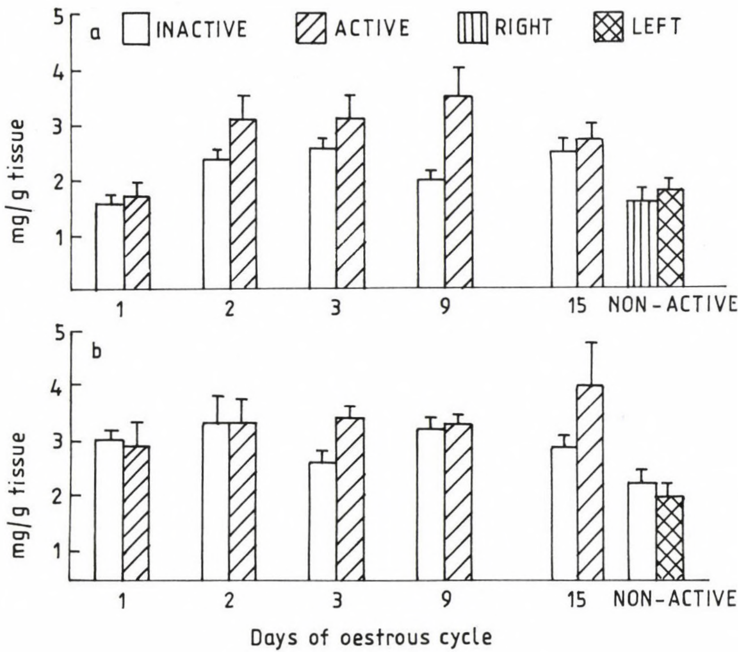

Fig. 4. Levels of nucleic acids (a) RNA and (b) DNA in ovarian tissue during different phases of the oestrous cycle. Values are mean ± SEM for 5 animals/group. * $P<0.05$ (comparison between the two ovaries)

Ascorbic acid

The averaged level of ascorbic acid was 264.16 ± 19.10 μg/g tissue for the active ovary and 228.33 ± 28.97 μg/g tissue for the inactive side (Fig. 5). Significant differences in ascorbic acid level during different phases of the oestrous cycle were found neither in active nor in inactive ovarian tissue. There were no differences in ascorbic acid during different stages of the cycle in the active ovary. Comparison between active and inactive tissue showed a significantly ($P<0.05$) different estimated ascorbic acid concentration only on D9.

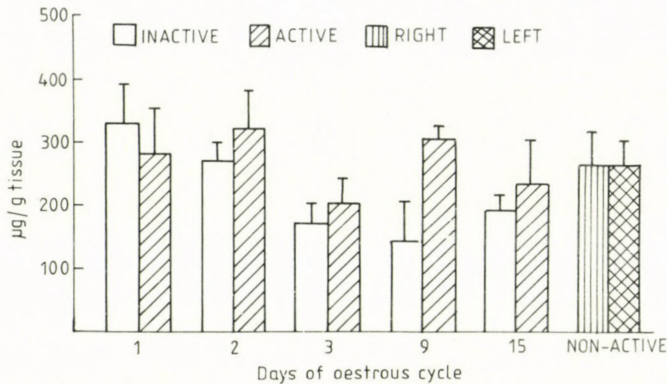

Fig. 5. Levels of ascorbic acid in ovarian tissue during different phases of the oestrous cycle. Values are mean ± SEM for 5 animals/group. * $P<0.05$ (comparison between the two ovaries)

Discussion

The increased total protein content found in this study could be attributed to follicular development under FSH stimulation during oestrus. The findings of Hutchinson and Robertson (1966) and Rao et al. (1989) support this assumption. Accumulation of protein during prooestrus to oestrus probably facilitates the formation of new cells. The decline in protein content from metoestrus to dioestrus may be due to the rapid atresia of immature follicles and to the declining CL. Higher level of total protein on D9 and D15 in the present study may be due to luteinization which leads to an increased volume of cells. Similar results were obtained by Rao et al. (1989). The elevated level of soluble proteins may be due to a higher synthesis of enzymes during ovulation. An increased enzymic activity of collagenase and plasminogen activator was reported by Rondell (1970) and Weiner and Kaley (1972). The lower level of soluble protein on D9 and D15 may be due to a higher utilization and less activity of enzymes on particular days of the oestrous cycle. The results obtained on alkaline phosphatase indicated that close to ovulation (D2), in the early luteal (D3) and follicular phase (D15) the cellular activities and metabolic transport may be

higher, hence alkaline phosphatase showed higher level during these periods. By splitting phosphoric acid from esters these phosphatase enzymes may participate in steroid hormone production, resulting in a higher activity on particular days. Goode et al. (1965) reported that in swine ovaries alkaline phosphatase was higher in the theca interna cells. During follicular development theca cells undergo extensive development, resulting in an active cellular process which elevates the alkaline phosphatase level of the theca interna cells.

The lower concentration of total cholesterol found at D9 and D15 may be due to the fact that during the mid-luteal phase more intensive progesterone synthesis takes place and, thus, a larger amount of cholesterol is converted into progesterone. At the same time, in the inactive ovary, in the absence of CL, progesterone synthesis is not so intensive and cholesterol content remains high due to non-utilization. The trophic stimulation of the ovary in many species results in a rapid loss of cholesterol ester from the gland (Flint and Armstrong, 1973). Sterol esters are the precursor for steroidogenesis and an inverse relationship exists between ester levels and progesterone (Robinson and Stevenson, 1971). Distinctly higher levels of free and esterified cholesterol were reported from ovarian tissue by many workers in ewe, doe, guinea pig, hamster and rabbit (Hoffman and Hofstetter, 1965; Flint and Armstrong, 1973; Hoftman and Fazer, 1973; Rao, 1985).

The conspicuous increasing trend of RNA recorded during oestrus might have been due to the fact that it participated in the synthesis of proteins required for the development of mature follicles and their ovulation. The present findings indicated that cellular activity and protein synthesis was reflected in the elevation of RNA and DNA on D1 to D15 of the oestrous cycle in the active ovary, but in the inactive ovary cellular activities were probably less expressed, which resulted in little variation in DNA as well as RNA concentration. In buffaloes the ovarian interstitial tissue contained a higher amount of RNA and DNA during the follicular and ovulatory phase (Janakiraman and Mehta, 1980).

The results obtained for ascorbic acid suggested that in the active ovary progesterone synthesis might be taking place. Along with an increase in progesterone concentration, ascorbic acid level also increased on D9 of the cycle. Progesterone level did not increase, in the inactive ovary, probably because its ascorbic acid concentration did not rise. Ascorbic acid

content was reported to be higher in buffalo ovaries during the luteal as well as the ovulatory phase, indicating that during these phases of the oestrous cycle ovarian tissues were probably more engaged with steroid hormone synthesis (Janakiraman and Mehta, 1988).

Acknowledgement

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COMPARATIVE PATHOGENICITY STUDY OF *Leptospira interrogans* serovar *pomona* STRAINS

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The comparative pathogenicity study of two *Leptospira interrogans* serovar *pomona* strains isolated from pig herds of different epizootiological status is reported. Using monoclonal antibodies (Mabs), the isolates were identified as *Leptospira interrogans* serovar *pomona*. The results obtained for the reference strain of *L. pomona* were identical with those of the two isolates, with the exception of monoclonal serum designated 61–7, which gave a 1:30 titre with the reference strain. In a pig herd comprising 1,000 sows, strain XVIII caused abortion in 300 animals. In the hamster pathogenicity test, this strain killed the hamsters on postinoculation (PI) days 5 or 6. The pathogen was reisolated from their organs. Strain XV did not cause abortion in a herd of 600 sows; it only elicited seroconversion in a proportion of the animals (26 out of 274 blood samples were positive, corresponding to a seropositivity rate of 9.5%). This strain did not kill the hamsters and could be demonstrated from their organs neither by isolation nor by histological examination. The blood serum of two pregnant sows infected with strain XVIII contained high titres of antibodies (1:1,600 and 1:3,200) already on PI day 10. The first urine sample of these animals, taken on PI day 10, also contained large numbers of leptospires. The pathogen was reisolated from urine samples taken at 10-day intervals and from the kidneys of the slaughtered sows. One of the two infected sows delivered four non-viable and a viable piglet. From the kidneys of the non-viable piglets, leptospires were demonstrated by histological examination. In contrast, none of the four pregnant sows inoculated with strain XV aborted. The sows did not produce antibodies detectable by the microscopic agglutination test (MAT). The pathogen could not be isolated from urine samples and from kidney samples taken from the sows at slaughter. Histological demonstration of leptospires from the sows' kidneys also failed.

Key words: *Leptospira interrogans* serovar *pomona*, pathogenicity, swine, abortion

Leptospirosis has long been recognized as an infectious disease causing severe economic losses in pig herds (Bohl et al., 1954; Ryley and Simmons, 1954; Bürki and Wiesmann, 1963). Economic losses result primarily from abortions, stillbirths, premature deliveries or from the birth of

non-viable piglets. Infected animals may shed the pathogen over a long period, even for a year, depending on the Leptospira serovar causing the infection (Faine, 1982).

In Hungary, leptospirosis of pigs is caused first of all by *Leptospira interrogans* serovar *pomona* and, to a lesser extent, *tarassovi* (Kemenes and Szemerédi, 1961). In addition to the above two serovars, abortion induced by *Leptospira interrogans* serovar *canicola* and *sejro* was also reported from this country (Kemenes, 1962; Kemenes et al., 1962). No verified or clinically apparent leptospiral disease caused by other known serovars has been diagnosed in Hungary as yet.

Infection by *L. pomona* can cause epizootic abortion among pregnant sows if infection takes place during the second half of pregnancy (Kemenes and Szemerédi, 1961). Besides this common and well-known epizootiological and clinical entity, however, in recent years we have observed several cases in which *L. pomona* infection of a previously infection-free herd was indicated exclusively by seropositivity.

Rather few data are available in the literature on infections causing exclusively seropositivity. Therefore, the aim of this work was to compare the pathogenicity of a *L. interrogans* serovar *pomona* strain isolated from an "atypical" case manifesting itself exclusively in seroconversion with that of a strain originating from a "classical" outbreak associated with abortions.

Materials and methods

Leptospira strains, experimental animals, and serology. The strains used in the experiment were isolated from pigs the sera of which had been sent to our Institute for serological testing and proved positive. The animals were treated with 4 ml Furosemide injection (Chinoin, Budapest; 10 mg furosemidum/ml) intravenously, then the urine was collected in a sterile flask. Immediately after sampling, 0.2 ml undiluted urine was inoculated into a semi-solid medium, 5 tubes per urine sample (Ellis and Thiermann, 1986). From the organs of the experimental animals, homogenates were prepared under aseptic conditions; 0.1 ml of the supernatant of these organ homogenates were inoculated into 5 tubes of the above medium each. The

cultures were incubated for 12 weeks. Upon the observation of leptospiral growth, subcultures were made in EMJH medium.

The isolated strains were determined using a monoclonal serum kit serving for the identification of serovars belonging to the *L. pomona* serogroup. The strain designated XV was isolated from a previously leptospira-free 600-sow herd in which 26 out of 274 serum samples tested in 9 months' time proved to be seropositive. However, no leptospira-induced abortion occurred in the herd, and no seroconversion of the entire herd had taken place. The other strain, designated XVIII, was isolated from a 1,000-sow herd which had also been free from leptospires previously. In that herd, almost 300 pregnant sows aborted within 2 months after infection.

After its isolation, strain XV was maintained in the semi-solid medium described by Ellis and Thiermann (1986) for 8 months until hamster infection and for further 4 months until use for the infection of experimental pigs, i.e. for a total of 12 months. Strain XVIII was maintained in the above medium for 6 months until hamster infection and for further 4 months after reisolation from the dead hamsters' organs. Thus, it was maintained in the medium for a total of 10 months before use for the infection of pigs.

In order to determine the virulence of the isolated strains, two golden hamsters (body mass: 50–52 g) each were infected with 3×10^7 leptospires derived from the 7-day culture of the isolates. At 35 days after infection, the surviving animals were killed, reisolation of the pathogen from their kidneys was attempted, and the specific antibody level of their sera was determined by the microscopic agglutination test (MAT).

In the pig experiments, gilts of 85–95 kg body mass were infected intravenously between day 60 and 65 of pregnancy. The sows originated from a herd free from brucellosis, leptospirosis, and Aujeszky's disease, and were serologically negative for *Leptospira pomona*.

Four sows were infected with strain XV: two sows (no. 1 and 2) received 2.8×10^7 leptospires while the other two (no. 3 and 4) were given 5.6×10^7 leptospires derived from a 7-day culture of the strain, in the form of injection into the v. cava anterior. On PI day 29, reinfection with strain XV was performed: sow no. 1 received 9.1×10^6 germs per os while sow no. 3 was given 1.3×10^8 germs intraperitoneally.

Two sows (no. 5 and 6) were infected with strain XVIII: sow no. 5 received 2.8×10^7 while sow no. 6 was given 5.6×10^7 germs intrave-

nously. At the time of infection, and subsequently at 10-day intervals up to farrowing, blood and urine samples were taken for isolation of the pathogen and determination of the antibody level. After the piglets had been weaned, the sows were slaughtered, and reisolation of the pathogen from their kidneys and uterus was attempted. Bacteriological examination of the kidneys, as well as their histological examination for leptospire detection using the silver impregnation technique were also performed.

The antibody level of the serum and urine samples was determined using MAT, starting from the 1:25 serum and 1:10 urine dilution. In the MAT, the strain used for inoculation was used as antigen. Blood samples giving at least 50% agglutination in the 1:100 dilution were considered positive. In the case of urine samples, the 1:10 dilution was regarded as positive.

The serum taken from sow no. 3 on PI day 19 was used for the growth inhibition test. The serum was sterilized by filtration and added to the EMJH medium at a ratio of 1 or 4%, respectively. EMJH medium containing 1 or 4% pig serum obtained from a herd free from brucellosis, leptospirosis and Aujeszky's disease, as well as EMJH medium free from pig serum, were used as control. To 10 ml medium prepared in that way, 1 ml of the 7-day culture of strain XV was added as inoculum. The germ count was determined after 4-day incubation at 29 °C.

Results

The results of tests performed with the isolated strains using monoclonal sera are shown in Fig. 1. Only sera designated 43–9 and 48–6 gave a reaction in the MAT; our isolates did not react with the other monoclonal sera. The reference strain of *L. pomona* differed to some extent from our isolates as it gave a reaction also with serum 61–7 in a low dilution (1:30).

Hamsters. No appreciable clinical signs were observed in the hamsters infected with strain XV. The hamsters were alert and had good appetite throughout the experiment. Attempts to recover the pathogen from the kidneys of hamsters killed on PI day 35 failed. The serum of both animals contained antibodies in a dilution of 1:200.

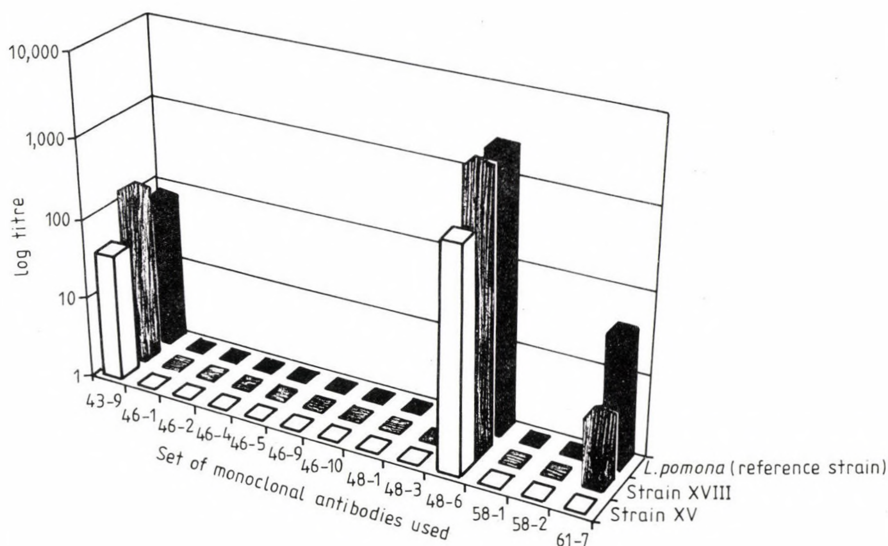

Fig. 1. Agglutination titres of monoclonal antibodies (Mabs) with field isolates and with the reference strain of serogroup *pomona*

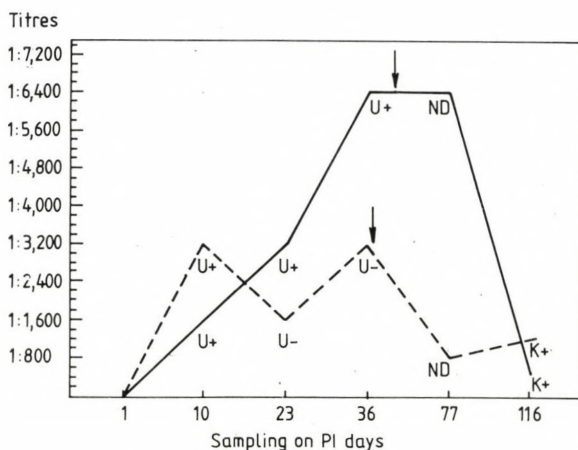

Fig. 2. Antibody titres of, and data of leptospire isolation from, sows infected with *Leptospira pomona* strain XVIII. Solid line: sow no. 5; broken line: sow no. 6; U: urine sample; K: kidney sample; ND: not determined; arrow: = time of farrowing

One of the two hamsters infected with strain XVIII died on PI day 5 while the other on PI day 6. The *L. pomona* strain was successfully isolated from the kidneys of both hamsters. Unfortunately, due to the sudden death of the hamsters, no blood samples could be taken for serological examination.

Pregnant sows. None of the four sows infected with strain XV aborted. The four sows delivered a total of 31 piglets between gestation days 111 and 117 (average litter size: 7.7). The piglets were healthy and viable. Five blood and five urine samples were collected between the sows' inoculation and farrowing. All these blood and urine samples proved negative for *L. pomona* antibodies. In the blood sample taken from sow no. 3 on day 10 after intraperitoneal reinfection a titre of 1:25 was measured.

Attempts to reisolate the pathogen from both the urine samples and the kidney and uterine mucosa samples taken at slaughter consistently failed. Similarly, histopathological examination of the kidney tissues by the silver impregnation technique yielded negative results, in the same way as the general bacteriological examination of the kidneys.

Of the two sows infected with strain XVIII, sow no. 6 delivered four poorly developed, non-viable and one viable piglet on gestation day 107. By histological examination of the kidneys of the non-viable piglets, leptospires were demonstrated in the lumen of the convoluted tubules. Sow no. 5 delivered six viable piglets on gestation day 112. Seroconversion of the two sows, the duration of leptospire excretion, and the results of leptospire isolation from the urine samples and from kidney samples taken from the sows at slaughter are presented in Fig. 2.

The sera of both sows contained high titres of antibodies already on PI day 10. In sow no. 5, the highest antibody level was found in the sample taken on day 36 (3rd blood sample after infection). In sow no. 6, a rapid initial elevation was followed by fluctuation of the titres. At the time of slaughtering the animals, 115 days after infection, high titres of antibodies were still present.

In the case of sow no. 5, *L. pomona* was successfully reisolated from all urine samples. In animal no. 6, leptospires were recovered from the first urine sample taken after infection. Concomitant bacterial infection of the samples taken during the further observation period rendered them unsuitable for examination. In the case of both animals, leptospires were success-

fully isolated from the kidney but not from the uterine mucosa samples taken at slaughter.

By examination of undiluted, non-centrifuged urine samples under a dark field microscope, the samples of sow no. 5 were found to contain numerous actively moving leptospires: in the sample taken on PI day 19, their number was 2.5×10^5/ml. The first urine sample taken from animal no. 6 after infection also contained many actively moving leptospires; however, the second and third urine samples were turbid and non-transparent. These latter samples contained many dead, immotile leptospires. On the average, 8 days were needed for the reisolation of leptospires from the positive samples.

In the growth inhibition test performed with the serum taken from sow no. 3 on PI day 19 and sterilized by filtration, 2×10^6 and 4×10^5 germs per ml were found in the cultures containing 1% and 4% serum, respectively, and 2×10^7 and 2.5×10^7 germs per ml, respectively, were present in the control cultures containing leptospira-negative serum at the above ratios. The germ count of the EMJH culture without pig serum was 2×10^7.

Discussion

Both *L. pomona* strains isolated from two different pig herds in this country were serologically identical. Using monoclonal antibodies both isolates proved to belong to *L. interrogans* serovar *pomona*. Although the strains showed only a slight difference with Mabs, they markedly differed from each other in their pathogenicity to hamsters and pregnant sows.

The results obtained for strain XV are not easy to explain. The blood samples taken on and after PI day 10 contained no antibodies demonstrable by MAT, and the pathogen could not be isolated from urine samples collected at the same time. These facts suggest that this strain could not survive and multiply in the experimental pig. This assumption seems to be supported by the observation that reinfection of two still seronegative animals on day 29 after the first infection was unsuccessful irrespective of its route (per os or intraperitoneal). This strain did not kill the hamsters and could not be reisolated from their organism. It induced only a low degree of seropositivity in the hamsters, which may perhaps be attributed to the

fact that the hamsters received a dose (expressed in germ count for body mass) several times higher than that given to the sows. The importance of antigen quantity is indicated by the result of intraperitoneal reinfection of sow no. 3. The dose administered at the first infection failed to elicit an antibody response detectable by MAT. A germ count twice as high as the first dose induced a weak antibody response in a titre of 1:25. The immune response was of short duration, as the antibodies were already absent from the subsequent sample taken 10 days later.

The time that elapsed between the isolation of strain XV and the infection of hamsters was 8 months, while that between isolation and the infection of sows was 12 months. For strain XVIII, the corresponding periods were 6 and 10 months, respectively. It has been reported in the literature that leptospira strains maintained on culture media over long periods lose some of their virulence (Faine, 1982). The hamster passage of strain XV proved to be unsuccessful, as this strain failed to kill the hamsters and could not be reisolated from their organs after the hamsters were killed. Despite this fact, our results can only partially be explained by data of the literature, partly because of the only 2-month difference existing between the two strains in the time of storage in the laboratory, partly because the two strains had caused basically different disease entities also in the herds of origin. One of them caused an outbreak characterized by the abortion of almost 300 sows, whereas the other induced only slight seroconversion restricted to a certain part of the herd.

Strain XV failed to elicit antibody production demonstrable by MAT in the sows but it proved to be an excellent antigen in the serological tests. This suggests that the antigenic determinants of a strain are not, or only partially, identical with its virulence factors. This assumption is consistent with the findings of Haake et al. (1991), who reported morphological dissimilarities and differences in the quantity of lipopolysaccharide-like substances between the virulent and avirulent strains of *L. grippotyphosa.*

Ballard et al. (1984) detected antibodies in the urine of experimentally infected pigs. The titre of these antibodies was the highest after the cessation of leptospiruria. In this study, we could not detect antibodies in urine samples from the two sows inoculated with strain XVIII. A possible explanation for this may be that even the last urine sample taken from these animals on PI day 36 contained large numbers of viable (sow no. 5) or dead (sow no. 6) leptospires.

By restriction endonuclease analysis (REA) of *L. pomona* isolates, Skilbeck et al. (1992) distinguished 3 genotypes. On the basis of their findings, they suggested that strains belonging to different genotypes might differ in their pathogenicity and host specificity. From the studies of Terpstra et al. (1987) it is known that genetically diverse *L. pomona*-like strains occur all over the world. Our results, which do not contain data from REA, seem to support the observations of the above authors in an indirect manner.

Further studies are needed to assess the correlation between REA and the pathogenicity of leptospire isolates.

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HUNGARY REMAINS FREE OF SCRAPIE AND BOVINE SPONGIFORM ENCEPHALOPATHY (BSE)

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Brains from 44 sheep and 43 cattle with CNS clinical signs not due to rabies virus infection were collected from diagnostic institutes throughout Hungary. The brains were examined for histological lesions diagnostic of scrapie/bovine spongiform encephalopathy (BSE) and all were found to be negative. These findings confirm that Hungary remains free of scrapie and BSE.

Key words: Scrapie, bovine spongiform encephalopathy

Historically, Hungary has only had two diagnosed cases of bovine scrapie. These occurred in two flocks in northeast Hungary in 1964. It was speculated although not proven that the disease entered one of these flocks via the importation of rams from outside the country. The disease spread to the second flock through movement of infected animals from the primary flock. Retrospective review of clinical disease in the flocks suggested that clinical scrapie had been present for 5 years. Both infected flocks were destroyed (Áldásy and Süveges, 1964).

Epidemiological studies based on clinical investigation confirmed that scrapie was eradicated from this geographic area and has not appeared in any other part of the country. Although the disease and its microscopic lesions were known by Hungarian diagnosticians, based on histopathological investigation of approximately 50–100 sheep brains annually there have been no subsequent diagnoses of scrapie in Hungarian sheep and there has never been a diagnosed case of bovine spongiform encephalopathy (BSE) in native or imported cattle in Hungary.

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In order to maintain the scrapie/BSE-free national status a monitoring program was initiated similar to the BSE monitoring program in the U.S.A. (Miller et. al., 1990). District diagnostic institutes were requested to submit brains from sheep and cattle with central nervous system signs that were not attributable to rabies virus infection (Fig. 1). Brains were sent to the Central Veterinary Institute for processing and evaluation. Two pathologists independently examined the histological sections.

Histological sections of brains from 44 sheep and 43 cattle with clinical signs of central nervous system disease were examined respectively for microscopic lesions of scrapie or BSE. Several brains of each species had characteristic lesions of listeriosis. A few sheep had one or two vacuolated neurons in the medulla and a few cattle had a few similarly vacuolated neurons in the mesencephalon. The aforementioned changes are considered incidental findings and have been previously reported in clinically normal sheep and cattle (Zlotnik and Rennie, 1957; Wells et al., 1987). It is assumed that the central nervous system clinical signs in the majority of the sheep and cattle resulted from metabolic or toxic conditions that do not produce microscopic brain lesions.

A diagnosis of scrapie is based on finding at least three of the following characteristics: spongiform vacuolation of the neuropil (status spongiosus), astrocytosis, single and multiple intracytoplasmic vacuoles that contain eosinophilic material in neurons, and neuronal necrosis. The diagnostic lesions of BSE are characteristically bilaterally symmetrical vacuolation of the neuropil of grey matter nuclei in the brain stem and intracytoplasmic vacuoles in neurons, most commonly in the dorsal nucleus of the vagus nerve, the reticular formation, and vestibular nuclei. Lesions are often present in the solitary tract nucleus and the spinal tract nucleus of the trigeminal nerve (Davis et al., 1991). There may also be mild astrocytosis and scattered necrotic neurons. Brain samples should include cross sections of thalamus, midbrain, pons, medulla and cervical spinal cord. In addition, a section of cerebrum, cerebellum and hippocampus should be examined.

Characteristic lesions of scrapie/BSE were not found in any of the brains collected for the survey. These findings confirm that Hungary remains free of scrapie and BSE.

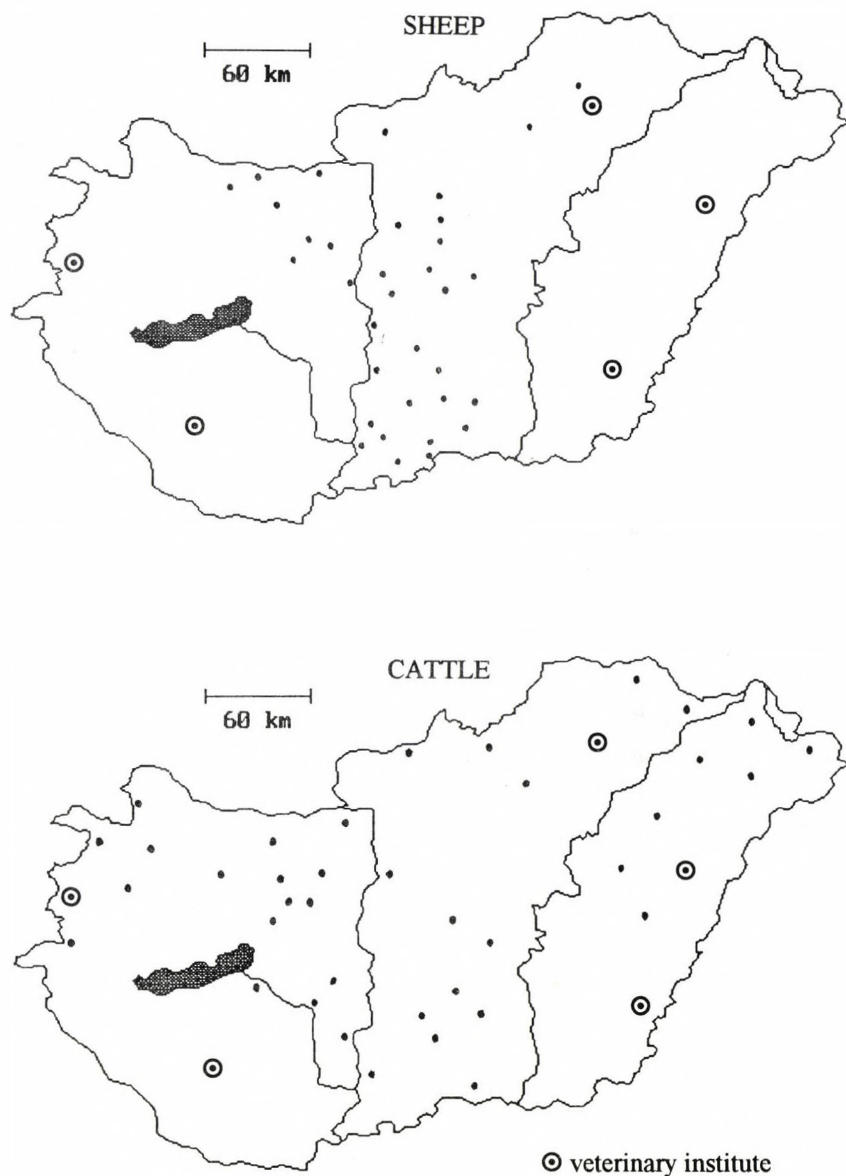

Fig. 1. Geographic location of flocks and herds of origin of submitted brains

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INFLUENCE OF PARENTAL ORIGIN, LITTER SIZE AND SEX ON THE FREQUENCY OF SPLAYLEG IN PIGLETS: A CASE REPORT

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The influence of parental origin, litter size and sex on the occurrence of splayleg was studied in 294 splayleg-affected litters with a total of 3,166 newborn piglets. Higher incidence of the disease was found in the litters sired by the SL-1 synthetic boar line, originating from crossings between German Landrace and Duroc breeds. At the same time, smaller litters were found more affected than the large ones ($P<0.01$). A higher number of affected males among splayleg piglets was found, but it did not reach the level of significance ($P<0.05$).

Key words: Splayleg, piglets, parents origin, litter size, sex

Splayleg, also known as myofibrillar hypoplasia, is a clinical disease of newborn piglets characterized by a temporary functional disorder with a reduced ability to adduct limbs, as the result of immaturity or dysmaturity of the skeleton muscle fibres (Ducatelle et al., 1986).

The morbidity and mortality rate within splayleg-affected litters can be high, and over 50% of deaths occur during the first 3 days after birth (Horugel and Lorenz, 1979).

The aetiology of the disease is still uncertain, but it appears to be multifactorial with hereditary (Vogt et al., 1984) and ill-defined environmental components (Curvers et al., 1989).

Investigations into the genetic background of the disease have noted a heritability coefficient (h^2) of 0.47 by half-sibs analysis (Sellier and Ollivier, 1982) and its occurrence within certain improved breeds, especially Landrace (Pour et al., 1984; Vogt et al., 1984; Montes and Herradora, 1986). Disadvantageous genetic correlations have also been found between the disease and traits such as body length, meat quality, growth rate and the number of live-born piglets in the affected litter (Jorgensen and Vestergaard, 1990; Svendsen et al., 1990; Essén-Gustavsson, 1992).

According to Jirmanova and Lojda (1985) and Tuček et al. (1985), environmental factors such as stress are also implicated in the aetiology of the disease. Splayleg syndrome can be a glucocorticoid myopathy induced by stressors during fetal development and/or a result of a nutritional shortage in sows during pregnancy.

The objective of this study was to compare the effect of parental (sow and boar) origin, litter size and sex on the occurrence of splayleg in piglets.

Materials and methods

The occurrence of splayleg was studied in 3,166 newborn piglets from 294 splayleg-affected litters with different combinations of final hybrids.

The 224 sows involved in our study represented three different genetic types: Slovak Large White (SLW) (a total of 40 sows), Slovak White Pork (SWP) (40 sows) and 131 SLW x SWP crossbred sows.

The impact of the sires' origin was studied using two synthetic lines. Synthetic line SL-1 consists of crossbred boars originating from crossings between German Landrace (GL) x Duroc (DU) (a total of 13 boars). Synthetic line SL-2 was the result of crossings between Belgium Landrace (BL) and Duroc (a total of 6 boars).

All the animals were fed standard commercial complete feed mixtures, and had uniform access to water. Housing and feeding technologies of "AGRA" type were employed, and artificial insemination of sows was used.

Statistical significance of differences between groups of animals was evaluated by Student's *t*-test (newborn piglets/splayleg piglets, presence of parental effect), and x^2 (Chi square) Test of Significance (sex ratio). Linear correlation and correlation coefficient were determined for relationship between litter size and incidence of splayleg.

Results

The influence of parental origin, sex and litter size on the occurrence of splayleg was studied in 294 splayleg-affected litters with a total of 3,166

newborn piglets. The total number of splayleg-affected piglets was 802, which represents 25.3%.

Table 1 presents the effect of parental origin on splayleg incidence: it shows that there are no significant differences (P>0.05) between the two synthetic lines (SL-1 and SL-2) of boars, and that the sows' origin (3 different genotypes) does not influence the occurrence of the disease either. However, it should be pointed out that although the boars' origin did not exert a statistically significant effect, clearly higher splayleg rates were found for litters sired by the SL-1 synthetic line where the average frequency of splayleg-affected piglets ranged from 25.9% to 26.3% per litter. At the same time, the frequency od splayleg-affected piglets in the litter sired by the SL-2 synthetic line was between 21.1% and 23.0%. Most splayleg-affected piglets (2.9 per litter) were seen among the SL-1 x SWP crossbreds.

Tables 2, 3 and 4 show the influence of litter size on the frequency of splayleg. The average litter size of splayleg-affected litters was 10.8 piglets, while the mean litter size for all sows at the farm was 9.3 piglets. A higher average number of all live-born piglets per litter was found in the litters sired by SL-1 boars (10.8) compared to SL-2 line (10.4), but the difference was not significant (P>0.05).

As shown in Fig. 1, the percentage of splayleg-affected piglets in litters sired by SL-1 boars was closely related to the litter size (r = —0.69; P<0.01), and decreased from 75% in small litters to 22% in large ones. The average percentage of splayleg for all the litters was 26%. In 77% of the affected litters most often there were 2 or more affected piglets per litter (P<0.05).

The number of males was higher among splayleg-affected piglets (ratio: 441 males/361 females), but the difference did not reach the level of significance P<0.05.

Table 1

Splayleg according to parental origin of piglets

Sires (line)	Sows (genetic type)								
	Slovak Large White			Slovak White Pork			Slovak Large White x Slovak White Pork		
	L	N/S	%	L	N/S	%	L	N/S	%
SL – 1 (GL x DU)	43	452/117	25.9	41	457/120	26.3	156	1693/439	25.9
SL – 2 (BL x DU)	13	135/31	23.0	13	142/30	21.1	28	287/65	22.6
Total SL–1+SL–2	56	587/148	25.2	54	599/150	25.0	184	1980/504	25.5

L: number of litter; N/S: total no. of newborn piglets/total no. of splayleg-affected piglets; %: percentage of splayleg

Table 2

Splayleg according to litter size

Boars (line)		Litter size (No. of piglets per litter)															Total
		4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	
SL–1 (GLxDU)	No. of litter	2	1	1	4	19	17	68	46	40	19	14	6	1	1	1	240
	No. of affected piglets	6	1	2	10	45	51	168	138	128	62	36	20	4	1	4	676
	% of affected piglets/ litter	75	20	33	36	30	33	25	27	27	25	18	22	25	6	22	26
						95% of litters											
SL–2 (BLxDU)	No. of litter			2	2	6	2	15	10	11	4	2					54
	No. of affected piglets			3	3	14	4	34	18	31	13	6					126
	% of affected piglets/ litter			25	21	29	22	23	16	23	25	21					22
						89% of litters											

Table 3

Litter size and frequency of splayleg according to boar origin

Boars (line)	No. of affected piglets	Litter size (No. of piglets per litter)															Total of litters
		4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	
		Number of litters															
SL–1 (GLxDU)	1		1		2	6	3	18	8	7	3	3	1		1		53
	2	1		1		7	5	23	12	10	2	6	2				69
	3				1	3	3	13	13	6	6	2	1				48
	4	1				2	5	10	9	10	4	1	1	1		1	45
	5				1			3		3	3	1					11
	6+					1	1	1	4	4	1	1	1				14
		Number of litters															
SL–2 (BLxDU)	1			1	1	3		4	5	1							15
	2			1	1	1	2	4	3	3	2						17
	3							6	1	4	1	2					14
	4					1		1	1	3							6
	5					1											1
	6+										1						1

Table 4

Distribution of splayleg-affected litters according to number of affected piglets per litter and parental origin of piglets

Boars (line)	Number of affected piglets/litter	Sows (genetic type)						Total	
		Slovak Large White		Slovak White Pork		Slovak Large White x Slovak White Pork			
		N	%	N	%	N	%	N	%
SL–1 (GL x DU)	1	11	26	12	29	30	19	53	22
	2	6	14	11	7	52	33	69	29
	3	15	35	6	15	27	17	48	20
	4	10	23	5	12	30	19	45	19
	5	1	2	1	2	9	6	11	6
	6					8	5	14	6
	No. of litters	43		41		156		240	
SL–2 (BL x DU)	1	4	31	4	31	7	25	15	28
	2	1	8	5	38	11	39	17	31
	3	5	38	3	23	6	21	14	26
	4	2	15	1	8	3	11	6	11
	5	1	8					1	2
	6					1	4	1	2
	No. of litters	13		13		28		54	

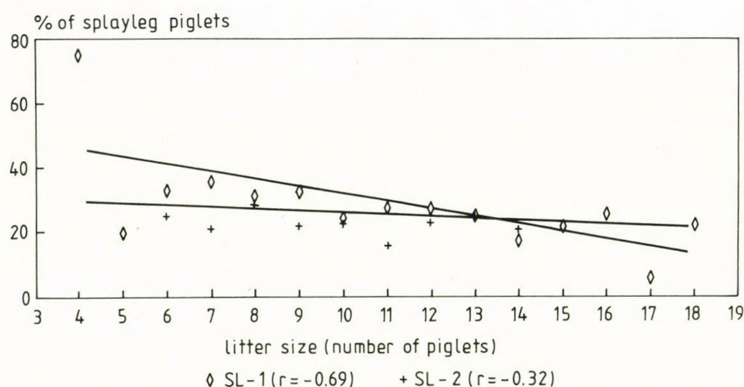

Fig. 1. Correlation between litter size and incidence of splayleg according to boar origin (r = correlation coefficient)

Discussion

Differences between the two synthetic sire lines found in the present study indicate that splayleg is to some extent genetically determined, which agrees with the results of Sellier and Ollivier (1982), Pour et al. (1984), Vogt et al. (1984), Lojda et al. (1985) and Montes and Herradora (1986). At the same time, the higher incidence of splayleg piglets sired by the SL-1 synthetic line shows the greater influence of the German Landrace breed genotype on the occurrence of the disease.

In contrast to the results of Sellier and Ollivier (1982), splayleg seems to be more a boar effect than a sow effect, as we did not observe significant differences ($P>0.05$) between the three different genetic types of sows mated by boars of either the SL-1 or SL-2 synthetic line.

With respect to sows' fertility, the largest mean litter sizes were produced by both groups of sires and SWP sows, while the lowest ones were seen in litters born of SLW sows. This is in agreement with relevant literature on the higher fertility of certain improved breeds, especially if improved by the Landrace breed (Montes and Herradora, 1986; Pour, 1986).

As opposed to the observations of Horugel and Lorenz (1979), Maas and Schultze (1979), Schnapperelle and Koch (1980), Sellier and Ollivier (1982), Pour (1985) and Van der Heyde et al. (1989), we found that, in litters sired by SL-1 boars, splayleg occurs more frequently ($P<0.01$) in

small litters than in the large ones. It is remarkable that such correlation was not found in litters sired by SL-2 boars.

In agreement with Lax (1971) and Bollwahn and Krudewig (1972) there were most often 2, 3 or more splayleg-affected piglets per splayleg litter. By contrast, Thurley et al. (1967) and Van der Heyde et al. (1989) mentioned an occurrence of only 1, sometimes 2 and seldom 3 affected piglets per splayleg litter.

Although other authors (Ward, 1978; Schnapperelle and Koch, 1980; Sellier and Ollivier, 1982; Pour, 1986; Van der Heyde et al., 1989) reported twice as many splayleg males than splayleg females in affected litters, we have not found significant differences (P>0.05) between the number of splayleg affected males and females, as also described by Hámori (1978) and Maas and Schultze (1979).

These results support the theory of Svendsen and Bengtsson (1980) that at least 2 clinical manifestations of splayleg can be distinguished: while the splayleg condition appears to be a sex-linked hereditary disease, the splayweak is a congenital disease not influenced by sex.

Finally, the occurrence of splayleg is significantly higher in small litters sired by SL-1 crossbred boars originating from crossings between German Landrace and Duroc. The number of splayleg-affected males and females was not significantly different. However, a slightly higher susceptibility to the disease was found in males.

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Sphaerospora siluri n. sp. (*Myxosporea: Sphaerosporidae*) IN THE KIDNEY OF THE SHEATFISH (*Silurus glanis*)

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Sphaerospora siluri sp. n. is a parasite of the sheatfish (*Silurus glanis*) cultured in Hungarian pond farms. Spores and pseudoplasmodia of the species inhabit the lumen of the renal tubules, attaching loosely to the tubular epithelium. Spores developing in monosporous pseudoplasmodia have an almost triangular shape.

Key words: European catfish or sheatfish, *Myxosporea*, new species, *Sphaerospora siluri*

More than 40 *Sphaerospora* species are known from freshwater and marine fishes. Most of them are found in the urinary system, primarily in cyprinid fishes. *Sphaerospora* infections have been found, however, also in fishes belonging to taxonomically different groups such as the salmonid *Salmo trutta* (Fischer-Scherl et al., 1986), the centrarchid *Lepomis gibbosus* (Li and Desser, 1985), the grouper *Epinephelus malabaricus* (Supamat-taya et al., 1991), the acipenserid *Acipenser ruthenus* (Baska, 1990), the percid *Gymnocephalus* spp. (Molnár, 1991) and the long nose skate *Raja rhina* (Arthur and Lom, 1985). Four species are known from the kidney of siluriform fishes. Among fish parasites of the Soviet Union Shulman (1984) recorded *S. dogieli* Schulman, 1962 from *Silurus soldatovi* and *S. schulmani* Allamuratov, 1966 from *Glyptosternum reticulatum*. From the American catfishes Lom et al. (1989) described *S. hankai* from *Ictalurus nebulosus* and Hedrick et al. (1990) described *S. ictaluri* from *I. punctatus*. Molnár and Baska (1992) suggested that the latter two species which infect phylogenetically closely related hosts might be synonyms.

Since the first extracellular developmental stages of sphaerospores were found by Csaba (1976), more data have become available on the development of *Sphaerospora* myxosporeans. Presporogonic developmental stages have been found in cyprinids (Kovács–Gayer et al., 1982; Körting,

1982; Csaba et al., 1984; Lom et al., 1983, 1985; Baska and Molnár, 1988) and in other fishes such as *Gasterosteus aculeatus, Ictalurus punctatus, Epinephelus malabaricus, Gymnocephalus schraetzer* and *Acipenser ruthenus* (Hedrick et al., 1988; Hedrick et al., 1990; Feist et al., 1991; Lom et al., 1991; Supamattaya et al., 1991; Molnár, 1991; Baska, 1990).

In the present paper, spores and presporogonic stages of a new *Sphaerospora* species are described from the kidney of the sheatfish (*Silurus glanis* L.), a cultured fish (known also as European catfish) that is well established in Hungarian pond farms and is being introduced to several Western European countries.

Materials and methods

From 1987 to 1993, 37 sheatfish (*Silurus glanis*) were collected from a fish farm close to the River Danube and from another farm close to the River Körös. The age of the fish varied from 3 months to 3 years. The fish were transfered live to the laboratory for later examination. They were killed by decapitation. Blood smears were prepared from each specimen, and pieces of the kidney, ureter and urinary bladder were examined fresh under a coverslip. The kidney was fixed for histological purposes if fresh examinations suggested the presence of spores or developmental stages. The kidney was fixed in Bouin's solution for 4 h, dehydrated in graded alcohols, embedded in paraffin, sectioned at 4 μm and stained with haematoxylin and eosin.

Results

Seven out of the 37 sheatfish examined proved to be infected by *Sphaerospora* stages. *Sphaerospora*-infected specimens were found both among month-old and three-year-old specimens, but infection was detected only in the spring and summer months. Four out of the 7 infected fish harboured spores in the renal tubules while the other 3 harboured only sporogonic developmental stages. Blood stages were found only in two specimens showing the heaviest infections. Description of the species based on 50 matured spores is as follows:

Sphaerospora siluri sp. n.
Host: *Silurus glanis* L.
Place of collection: Fish farms of Szarvas and Százhalombatta close to the River Körös and River Danube, respectively
Site of infection: Renal tubules of the kidney

The spores (Figs 1 and 2) and pseudoplasmodia occurred in the lumen of the convoluted tubules and, less often, inside the Bowman's capsule. The youngest pseudoplasmodia were composed of a primary cell and a secondary cell (sporoblast). All pseudoplasmodia were monosporous. The sporoblasts had 6 nuclei which together formed one spore. Pseudoplasmodia containing 6 cells of the sporoblast and one cell of the primary cell were amorphous, rounded or ellipsoidal. Rounded pseudoplasmodia measured 10–13 μm in diameter, while ellipsoidal ones were 15–17 × 11–12 μm.

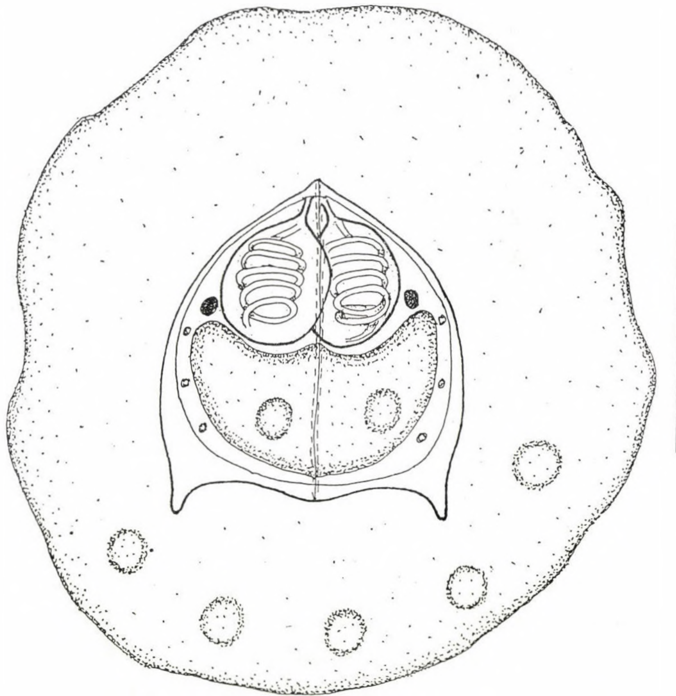

Fig. 1. Schematic illustration of a *Sphaerospora siluri* n. sp. spore within the pseudoplasmodium. Bar = 5 μm

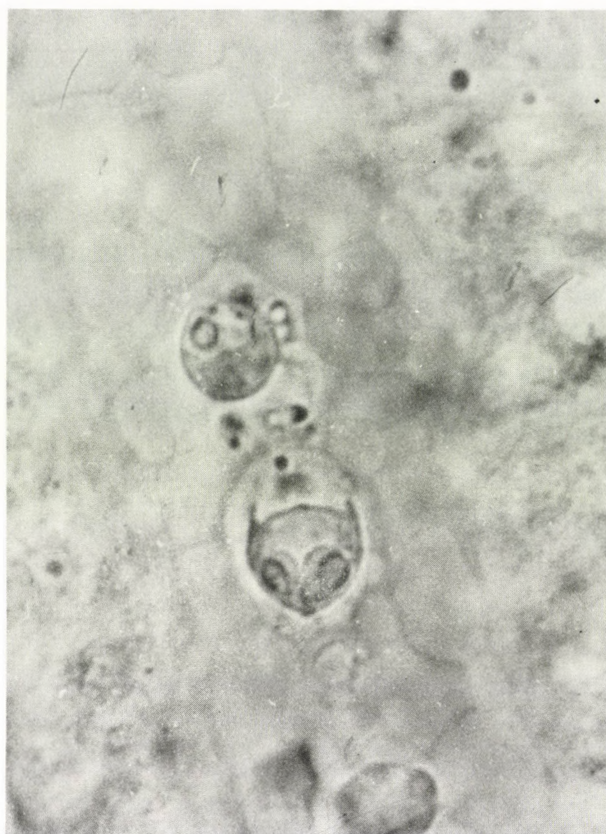

Fig. 2. S. siluri spores in the renal tubules. Monosporous pseudoplasmodia harbour only one spore. One of the spores have typical ear-like protrusions. × 2,100

Spores in sutural view are almost triangular in appearance having two lateral ear-like protrusions. Sutures are prominent showing small protrusions at the base and at the posterior end. Length of the spores 6.8 (6.6–6.9) and their width 6.8 (6.7–6.9) µm. The thin shell has a smooth surface. No mucous envelope was found. Two equal, subspherical polar capsules measure 3.1 (2.8–3.4) µm in diameter. Polar filaments with 5 to 6 turns aligned almost perpendicular to the longitudinal axis of the capsule.

Early extrasporogonic blood stages resembling C-stages of the common carp were found only in two fish. Csaba (personal communication), however, frequently found these stages in *Sphaerospora*-infected sheatfishes.

In histological sections only moderate infections were found. Pseudoplasmodia of different developmental stages were found only in the anterior segments of the convoluted tubule where they attached loosely to the tubular epithelium. No pathological changes were observed.

Remarks: by having monosporous pseudoplasmodia, ear-like protrusions and prominent sutures on the spores this species resembles both *S. hankai* and *S. ictaluri*, but it differs from them by the larger size of spores and by having 5 to 6 coils in the polar capsule. Moreover, the hosts are phylogenetically distant from each other. The spores of *S. dogieli* and *S. schulmani* are about the same size but the shape of the spores is different.

Discussion

The sheatfish (European catfish) is an economically important fish in Hungarian pond farms. Its culture technology has been developed mostly by Hungarian fish culturists. Relatively little is known about parasitic diseases of this fish species. Up to the present, detailed information has been available only on *Ancylodiscoides vistulensis*, a monogenean parasite causing gill disease of fry (Molnár, 1980). *Sphaerospora* spp. are potential pathogens of fishes, and their presporogonic stages may cause severe diseases like swimbladder inflammation of the common carp and PKD of salmonids (Csaba et al., 1984; Kent and Hedrick, 1985). The intensity of infection found by us in sheatfish was relatively low, and no diseases were attributed to the infection found. Even blood stages characterizing heavy infections were detected only in one case. These stages corresponded to typical blood stages having at most 8 secondary cells in the primary cell. In catfish infected with *S. ictaluri*, however, Hedrick et al. (1990) found stages resembling swimbladder forms of the common carp in different organs, and postulated a connection between proliferative gill disease and *Sphaerospora* infection in channel catfish. At the present time *Sphaerospora siluri* infection of the sheatfish cannot be associated with diseases of the species.

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EFFECT OF DECREASED OXYGEN CONTENT ON EELS *(Anguilla anguilla)* INFECTED BY *Anguillicola crassus* (NEMATODA: DRACUNCULOIDEA)

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The survival chances of eels with *Anguillicola crassus* infection of varying intensity and with varying pathological changes were studied in an experimental system devoid of fresh oxygen supply. Eels most severely affected by anguillicolosis died first, while those with less expressed pathological lesions tolerated sublethal oxygen levels for a longer time. Findings were similar at 20–21 °C and at 27–28 °C; at 27–28 °C, however, the fish required a higher oxygen content to survive. The experiments demonstrate that *Anguillicola* infections substantially impair the eels' natural resistance and, under unfavourable environmental conditions, may lead to their death.

Key words: Eel, *Anguillicola crassus*, oxygen deprivation, pathogenicity

Since in the late 1980's the nematode *Anguillicola crassus* Kuwahara, Niemi et Itagaki, 1974 was introduced to Europe, dozens of papers have discussed its rapid spread, development and prevalence. However, relatively few researchers have dealt with the parasite's effect on the fish organism and with its pathogenicity. Of the latter, Boon et al. (1989, 1990*a*, 1990*b*) and Höglund et al. (1992) studied the effect exerted by the parasite on the eel's blood composition, while Sprengel and Lüchtenberg (1991) reported that parasite-infected eels were characterized by a reduced swimming speed. The observation of Thomas and Ollevier (1992), i.e. that more severely infected eel specimens are more easily sucked in by the water-pipes of power stations than those with a less intensive infection, also belongs to this category. Only a single work (Molnár et al., 1993) furnishes detailed information about the histopathology of *Anguillicola* infection, but data on the helminths' pathogenic effect exerted on the swimbladder and on occasional mortalities can be found also in the works of Egusa (1979), Hartmann (1987), Liewes and Schaminee-Main (1987), Mellergaard (1988), Boon et al. (1989), van Willigen and Decker (1989),

Haenen et al. (1989), Banning and Haenen (1990) and Kamstra (1990). Similar data concern infections of eels with *A. globiceps* (Yamaguti, 1935) and with *A. novaezelandiae* (Sarti et al., 1985). To date, only one paper (Molnár et al., 1991) has been published on mass mortality caused by *A. crassus*.

In the summer of 1991, a mass and selective mortality occurred among eels of Lake Balaton in Hungary. This mortality recurred, though in a less severe form, in 1992. About 200 tons of eel died in 1991 and 40 tons in 1992. Our research team attributed this mortality to the extremely severe *Anguillicola* infection and the resulting swimbladder damage. As this opinion was received sceptically by Hungarian specialists unfamiliar with fish pathology, we conducted experiments to determine the influence exerted by adverse environmental conditions on the survival rate of eels whose physiological capacity and natural resistance had been impaired by *Anguillicola* infection. As a first step, the survival chances of eels affected with *Anguillicola* infection of varying intensity were studied in an environment of low oxygen content.

Materials and methods

Eels (*Anguilla anguilla* L.) derived from Lake Balaton and naturally infected by *A. crassus* were used in the experiments. The eels were 17–72 cm long and had been kept in the laboratory for a few days before the experiment. Each treatment group comprised 10 fish. The fish used in the individual experiments had always been derived from the same place and, as far as possible, they were of nearly the same size. The experimental fish specimens were placed in 10-litre aquaria filled with water to repletion and covered with a glass plate to prevent fresh oxygen supply. During the experiments, water temperature and oxygen content were recorded quarter-hourly. Water temperature was measured with a common internal water thermometer. Oxygen content of the water was measured with "Aquacheck", an instrument developed by the Radelkis Co-operative (Budapest) and routinely used in the Hungarian farm-pond practice for measuring water pH and oxygen level. The instrument measures the percentage of oxygen dissolved in water. From that value, the concentration of dissolved oxygen (in mg/litre) can be calculated with the help of a table if water temperature is known. Two temperature ranges were used. In ex-

periments conducted in the spring, water temperature of the aquaria was adjusted to 20–21 °C while in the summer experiments water temperature was kept at 27–28 °C. Eels that died of anoxia were removed from the water in the order and at the time of death. The oxygen level measurable at the time of death was determined. If the last two eels of a group did not die shortly after the others, they were killed with an overdose of the hypnotic MS-222.

The dead eels were then subjected to full parasitological dissection. Particular attention was paid to the swimbladder lesions. The number and size of worms found in the swimbladder, the developmental stage and number of larvae burrowing in the swimbladder wall, the thickness of the swimbladder wall, the status of its mucous membrane, the occurrence of foci, pigment deposits or abscesses in the swimbladder wall, and the quantity and quality of fluid released from disrupted worms present in the swimbladder lumen were recorded.

Both the adult and juvenile specimens of *Anguillicola* and the 3rd and 4th stage larvae occurring in the swimbladder wall were detected with the help of a stereomicroscope; however, only the stages found in the swimbladder lumen were collected. Since the size of eels used in the different experiments was not uniform and the worms represented different stages of development, the number of worms found in the swimbladder is a less suitable indicator of the severity of infection. Therefore, we also used the term "quantity of worms" to indicate the extent to which the swimbladder was filled with worms. In cases marked *** the swimbladder was either completely filled with worms or it was at least ¾ full, in cases marked ** it was filled approximately to half, while the score * represented cases when only a few adult worms or several juvenile stages were present in it. The wall thickness of the swimbladder was measured in mm. In the majority of cases this value represented the wall thickness of the organ cut through in the middle in transverse direction. In some cases, however, only one or both ends of the swimbladder were thickened. In those cases wall thickness was given as a mean value. Wall thickness values exceeding 3 mm corresponded to severe, those between 1.5 and 3 mm to moderate, while values between 1.0 and 1.5 mm to mild thickening. Although a more than >0.3 mm wall thickness of the swimbladder can usually be attributed to infection, swimbladder walls less than 1 mm thick were considered thin in this study. The data are summarized in Tables 1 and 2.

Table 1

Oxygen deficiency tolerance of eels affected by *Anguillicola crassus* infection of varying severity at 20—21 °C

No. and date of exp.	Order of deaths	Length of eel (cm)	Time of death[1]	O_2 content of water (mg/l)[2]	Swimbladder lesions						Worms in swimbladder wall		Larvae in swimbladder wall		Severity grade[3]
					Swimbladder wall thickness (mm)				Other lesions						
					3–7	1.5–3	1–1.5	<1	Focus in wall	Fluid in lumen	number	quantity	4th stage	3rd stage	
I 26.2	1-2	24	3'40	0.99	*						-	-	-	-	1
	1-2	30	3'40	0.99			*				11	**	3	2	2
	3	43	4'10	0.99		*				*	3	*	2	-	3
	8-9	36	7'10	0.99			*			*	-	-	3	-	9
	8-9	31	7'10	0.99				*			-	-	-	-	10
	10	45	7'50	0.99			*				1	*	4	2	8
II 27.3	1-2	35	4'00	1.11			*				-	-	-	-	2
	1-2	25	4'00	1.11				*			1	*	1	-	3
	3	44	4'50	1.01				*			10	*	3	5	1
	8	30	5'40	1.01				*			-	-	-	-	9
	9	33	7'00	1.01				*			-	-	2	-	8
	10	50	killed	1.01				*			-	-	-	-	10
III 07.5	1	78	5'00	1.33		*					30	***	20	10	2
	2	78	5'20	1.33		*				*	9	**	100	20	3
	3	68	6'30	1.05		*				*	38	***	35	70	1
	8	62	10'20	0.90			*		*		-	-	-	-	8
	9-10	56	killed	0.90				*			-	-	-	-	9-10
	9-10	58	killed	0.90				*			-	-	-	-	9-10

Table 1 continued

No. and date of exp.	Order of deaths	Length of eel (cm)	Time of death[1]	O_2 content of water (mg/l)[2]	Swimbladder lesions						Worms in swimbladder wall		Larvae in swimbladder wall		Severity grade[3]
					Swimbladder wall thickness (mm)				Other lesions						
					3–7	1.5–3	1–1.5	<1	Focus in wall	Fluid in lumen	number	quantity	4th stage	3rd stage	
	1	70	2'00	1.15	*						68	* * *	10	80	1
	2	72	5'10	1.06		*					56	* * *	40	15	2
IV	3	72	7'15	0.89		*					13	*	6	14	3
11.5	8	70	9'30	0.89			*				2	*	4	10	6
	9-10	66	killed	0.89				*			6	*	2	20	7-8
	9-10	68	killed	0.89			*				4	*		30	7-8
	1	73	4'30	1.06	*						59	* * *	20	115	1
	2	51	6'00	0.97	*						-	-	5	200	2
V	3	61	6'15	0.97	*				*		-	-	-	-	3
12.5	8	65	8'20	0.89			*		*		7	-	15	20	7
	9-10	69	killed	0.89			*		*		-	-	10	10	8
	9-10	59	killed	0.89				*			-	-	-	-	10

[1] Time of death after oxygen deprivation
[2] O_2 content of water at time of death (mg/l)
[3] Severity grade by gross pathological findings

Table 2

Oxygen deficiency tolerance of eels affected by *Anguillicola crassus* infection of varying severity at 27—28 °C

No. and date of exp.	Order of deaths	Length of eel (cm)	Time of death[1]	O_2 content of water (mg/l)[2]	Swimbladder lesions						Worms in swimbladder wall		Larvae in swimbladder wall		Severity grade[3]
					Swimbladder wall thickness (mm)				Other lesions						
					3–7	1.5–3	1–1.5	<1	Focus in wall	Fluid in lumen	number	quantity	4th stage	3rd stage	
	1	36	1'00	2.24	*				*		-	-	-	-	1
	2-3	23	1'15	1.60							-	-	-	-	2
VI	2-3	32	1'15	1.44							1	*	-	-	3
03.8	8	39	2'20	1.44				*			7	*	-	2	8
	9	27	2'20	1.44				*			-	-	1	10	10
	10	50	4'00	1.44				*			4	*	7	7	9
	1-2	29	1'05	2.24	*						-	-	-	-	1
	1-2	29	1'05	2.24	*				*		-	-	-	-	2
VII	3	31	1'45	1.76	*						-	-	-	-	3
06.8	8	38	3'00	1.60							-	-	-	-	8
	9	33	4'00	1.60			*				1	*	-	-	9
	10	35	killed	1.60				*			-	-	-	-	10
	1-2	43	1'30	2.56	c						-	-	-	-	1
	1-2	47	1'30	2.56	*						-	-	-	-	2
VIII	3	44	1'35	2.27	*					*	-	-	-	-	3
08.12	8	53	4'00	2.27			*				-	-	1	-	9
	9	51	4'10	2.27				*			3	**	4	8	8
	10	50	4'20	2.27			*				-	-	-	-	10

Table 2 continued

No. and date of exp.	Order of deaths	Length of eel (cm)	Time of death[1]	O_2 content of water (mg/l)[2]	Swimbladder lesions						Worms in swimbladder wall		Larvae in swimbladder wall		Severity grade[3]
					Swimbladder wall thickness (mm)				Other lesions						
					3–7	1.5–3	1–1.5	<1	Focus in wall	Fluid in lumen	number	quantity	4th stage	3rd stage	
IX 13.8	1	23	2'05	2.40		*					1	*	-	-	2
	2	24	2'03	2.24	*						2	*	1	-	1
	3	49	2'35	2.24		*					-	-	-	-	3
	8	17	2'40	2.24			*				-	-	-	-	8
	9	23	3'30	2.24				*			-	-	-	1	10
	10	41	3'40	2.24			*				-	-	1	-	9
X 31.8	1-2	19	1'10	2.40		*					-	-	-	-	3
	1-2	28	1'10	2.40			*				6	**	4	-	2
	3	19	1'20	2.30		*					-	-	1	-	4
	8	30	2'10	2.30				*			2	*	-	-	8
	9	30	killed	2.30				*			-	-	-	2	9
	10	31	killed	2.30				*			-	-	-	-	10

[1] Time of death after oxygen deprivation
[2] O_2 content of water at time of death (mg/l)
[3] Severity grade by gross pathological findings

Two different rankings were used for evaluating the results. The chronological order of death was determined and the cases were ranked by severity according to the gross pathological findings. In determining the severity grade, less importance was attached to the number of worms and larvae present. The grade of severity was determined primarily on the basis of swimbladder wall thickness, the number of foci present in the swimbladder wall and the quantity of fluid observed in the lumen of the swimbladder. In cases marked *** and **, and also in the case of intensive larval infection, however, the number of worms or larvae was also taken into consideration when establishing the severity scores. Although 10 eels were always examined, only data on the three eels that died first and those of the last 3 survivors are included in the tables for a better demonstration of the differences.

Although a few *Pseudodactylogyrus* monogeneans were often found on the gills of the experimental eels, and *Eimeria anguillae, Myxidium giardi* and *Myxobolus portucalensis* infections of low intensity were also observed in the intestine, parenchymal organs and on the fins, respectively, no substantial parasitosis was present which could have disturbed the experiments. In the majority of the dissected eels, *Aeromonas* and *Pseudomonas* bacteria were isolated from the swimbladder; with the exception of one fish, however, these bacteria did not produce clinical signs.

Results

The data presented in Tables 1 and 2 indicate that there is a rather close correlation between the severity of *Anguillicola* infection and toleration of hypoxic environment by the eels. In 9 out of the 10 experiments, the three fish judged to be the most severely affected on the basis of the gross pathological findings were among those three eels which died first. In 8 out of the 10 experiments, the three fish considered to be the least affected survived for the longest time in the hypoxic environment.

Table 1 shows that at a water temperature of 20–21 °C the eels will die if the oxygen content is 0.89–1.15 mg/l. However, significant differences are demonstrable between the less affected and the more severely affected specimens in the time of tolerating this low oxygen content. In the case of an oxygen content higher than that (1.33 mg/l) only two fish with

extremely severe infection died at the above temperature (Table 1, experiment 3).

In the five experiments summarized in Table 2 water temperature was adjusted to 27–28 °C. In that case, deaths occurred at an oxygen content of 1.44–2.56 mg/l. Also in that case, eels with a markedly thickened swimbladder wall died at the highest oxygen values (2.56 mg/l).

Only experiments no. 4 and 5 seem not to fit in this picture: namely, in these experiments eels with a less severely damaged swimbladder died earlier than fish judged to have more severe lesions. It should be mentioned, however, that these eels showed the signs of incipient bacterial infection. The "first three" data of experiment 9 also differ from the results expected: here the fish judged to be the most severely affected died later than the three others following it in the order of severity. In that experiment, however, severity grades were difficult to establish because of the lower intensity of infection and the less expressed swimbladder lesions.

Discussion

No unequivocal data are available on the physiological effect and pathogenicity of anguillicolosis in eels. At the same time, Sprengel and Lüchtenberg (1991) have reported that the presence of already a few worms markedly reduced the swimming speed of eels. Thus, helminth infection affects the physiological capacity of the fish. The results of the present experiments demonstrate that this effect can sometimes be very substantial and may impair the eel's natural resistance to such an extent that the fish becomes vulnerable to environmental stress factors and eventually dies.

The experiments have confirmed our earlier statements (Molnár et al., 1993) that the severity of damage is determined by the infection-induced thickening of the swimbladder wall rather than by the number of worms present in the swimbladder. Eels having a thick swimbladder wall and infected by large numbers of worms can be considered the most severely affected. A stage almost as severe as that is spoken of when worms and larvae are no longer present in the swimbladder as a result of the host reaction, but the swimbladder shows anatomical deformities and physiological dysfunctions resulting from the infection. According to the results

obtained by Molnár et al. (1993), infection by larvae and worms, the appearance of a fluid containing blood and worm debris in the swimbladder lumen as a result of worm disruption, the subsequent serous and fibrous thickening of the swimbladder wall, and the formation of foci around the necrotic larvae should be considered the stages of the same process. Within that process, the stage characterized by the absence of worms from, and the presence of severe lesions in the swimbladder represents a phase when the course of the disease is decided: either regeneration of the swimbladder will take place or the fish will die. According to Kamstra (1990), disruption and full atrophy of the swimbladder are common in the final phase of the disease. In that case, stress factors probably do not play a role, and the eel dies even in the absence of external factors impairing its natural resistance. However, at the peak of infection or at the time of helminth elimination, when the chances of regeneration and death are approximately equal, the presence of adverse external conditions markedly influence the chances of survival, as it has been shown in these experiments.

Practically no eel free from *Anguillicola* infection can be found in Lake Balaton. Therefore, in my experiments the eels free from worms and larvae represented specimens which had recovered from infection and were not yet affected by reinfection. At the same time, in the rivers of Western and Northern Europe characterized by a lower intensity of infection, the negative fish include both eels which have never been infected and those which have recovered from infection, are not infected by helminths but show lesions caused by the helminthosis. If fish of such diverse history are used in experiments as controls, this may lead to false results. Presumably this is why Boon et al. (1989, 1990*b*) and Höglund et al. (1992) found only minor differences between the infected and the control fish in haematocrit and serum protein profiles. Similarly, Möller et al. (1991) observed only minor differences between infected and control eels in body condition and liver somatic index. From the tables of their paper it is apparent, however, that the highest standard deviation was observed for the control group in both the body condition factor and the liver somatic index. This can probably be attributed to the fact that the authors evaluated the intensity of *Anguillicola* infection merely by the number of worms present, disregarding the pathological changes of the swimbladder, and — besides the yet uninfected fish — included in the "control" group also the eels representing the most advanced stage of infection, i.e. those which had just got rid of

worms and showed various stages of swimbladder regeneration. Studies performed by Boon et al. (1990*a*) using carefully selected controls, however, show that under experimental conditions a significant change in haematocrit and plasma protein values can be observed in eels infected by larvae.

The present experiments clearly demonstrate that *Anguillicola* infection markedly impairs the eels' physiological capacity and natural resistance. In the presence of any stress factor harmless to healthy fish, this infection increases the chances of mortality. (In our experimental model, high temperature and hypoxia were used as stress factors). The data obtained support our view that the mass mortality that occurred among eels of Lake Balaton in 1991 and 1992 was basically due to intensive, clinically apparent *Anguillicola* infection. This does not amount to saying that hypoxia certainly played a role in the observed mortality. Artificially produced oxygen-deficient environment only simulated a possible stress factor which may develop in the lake at any time. In these experiments, at a higher water temperature the fish became affected even if the oxygen content was higher. This is suggestive of a close correlation between temperature as a stress factor and the summer mortality of eels.

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EFFICACY OF BUTALEX IN FIELD CASES OF BOVINE THEILERIOSIS — SHORT COMMUNICATION

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Lactating cows, heifers, calves and young bulls suffering from naturally occurring bovine tropical theileriosis were treated with Butalex (buparvaquone). As a result of treatment, the clinical signs disappeared and the previously depressed milk yield of cows increased. No mortality was recorded and all the animals recovered.

Key words: Theileriosis, Butalex, treatment, haematology

A new compound, 2-trans (4-t-butylcyclohexyl) methyl 1-3-hydroxy-1,4 naphthoquinone, designated Butalex (buparvaquone), has recently been developed by Coopers Animal Health Ltd. (U.K.) for the treatment of bovine theileriosis. The drug has been claimed to be effective against experimental infections due to *Theileria annulata* (Ankara) (McHardy et al., 1985; 1987), *T. parva* (Muguga) (Morgan and McHardy, 1986), *T. annulata* (Hissar) (Dhar et al., 1986, 1987), *T. annulata* (Izatnagar) (Sharma and Mishra, 1990) and clinical cases of theileriosis (Mishra et al., 1990). The efficacy of Butalex against naturally occurring bovine theileriosis was tested in the field in the present investigation.

On BHEL dairy farm (Ranipet, India), lactating cows, heifers, calves and young bulls had been suffering from theileriosis. Blood and lymph node smears prepared from them revealed the presence of *Theileria annulata* schizonts or piroplasms. All animals included in the present study exhibited elevated body temperature ranging from 103.8 to 107 °F with an average of 105.5 °F and haemoglobin values as low as 5.8 g/100 ml with an average of minimum value of 6.7 g/100 ml of blood.

In lactating cows, the average milk yield of 16.8 kg per day dropped to 7 kg per day. The visible mucous membranes became icteric and rumination was either reduced or totally suspended. The cows had anorexia,

dark-coloured urine, and pellety or dry dung coated with mucus. The animals also exhibited dyspnoea and coughing. One cow revealed nervous symptoms, which included frequent shaking of the head, pressing the head against the manger and slight droppings of saliva. Some of the animals exhibited lacrimation also. In a young bull of about 1½ years of age, frequent micturition was also observed with extreme dullness.

All the sick animals were given Butalex at the dose rate of 2.5 mg/kg body mass intramuscularly. Symptomatic treatment, using analgin for bringing the body temperature down, was given to some of the animals. Anorexon, liver stimulant, Belamyl, multivitamin injectable were also given as supportive therapy. Animals with complete inappetence were given rice gruel. The deteriorating condition of some of the animals also required the intravenous administration of 20% dextrose. Besides all this treatment and supportive therapy, two out of the 11 animals had to be given a second dose of Butalex.

The average body temperature of the treated animals returned to 101.6 °F on post-treatment day 4 when they also started taking food. The treatment of lactating cows resulted in an improvement of the milk yield (which was reduced during the pretreatment period) from post-treatment day 2. By day 8 to 10 the milk yield reached 12 kg per day.

The average haemoglobin value of 6.7 g/100 ml of blood (ranges: 5.8–8.2) improved after treatment to an average of 8.6 (ranges: 7.2–10.2) in 4.6 days (average) after treatment. By post-treatment day 4 the appetite and general body condition of the animals also improved, the mucous membrane returned to its normal colour, and urine became straw coloured. There was marked improvement in the general condition, dyspnoea and coughing. All animals showed good recovery. No death was reported among the treated animals. From the observations made during the treatment trial it can be concluded that Butalex is highly efficacious in animals suffering from clinical theileriosis in field cases.

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CONCURRENT ORAL ADMINISTRATION OF MONENSIN AND SELENIUM TO BROILER CHICKENS: EFFECTS ON CONCENTRATION OF DIFFERENT ELEMENTS IN THE LIVER*

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Broiler chicks were kept on feeds amended by the addition of 240 mg monensin and 15 mg selenium with or without 200 mg vitamin E/kg. After 12 days, birds in different groups were orally administered three doses of 250 mg monensin and 5 mg selenium/kg body weight. In the second experiment, after four weeks of adaptation on amended feeds, similar groups were orally administered 40 mg monensin and 1 mg selenium/kg body weight on alternate days for four weeks. Monensin increased the liver iron level. Selenium increased the hepatic levels of selenium and iron while variable degrees of depression occurred in copper, zinc, manganese and magnesium levels. Concurrent administration of monensin and selenium significantly increased the liver selenium levels. A marked decrease in body weight and increased mortality were recorded due to concurrent administration of monensin and selenium.

Key words: Selenium, monensin, chicken, liver minerals

Selenium is an essential element in animal and human nutrition. Several enzymes in prokaryotic cells are dependent upon selenium; of them, glutathione peroxidase (GSH-Px) is the most important. Selenium compounds are widely administered to domestic animals for the treatment and prophylaxis of unthriftiness, reproductive disorders and also as growth promoters. However, due to the narrow margin between therapeutic and toxic doses, accidental overdosage in livestock often results in severe

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losses (Anderson et al., 1985; Castle et al., 1985; Hill et al., 1985). Selenium interacts with other metals and nutrients (Underwood, 1971) and improves the copper status of sheep in copper-deficient areas (Thomson and Lawson, 1970). Its presence in the diet partially protects rats from toxic levels of cadmium (Reddy et al., 1978) and paraquat (Omaye et al., 1978). The effect of toxic levels of selenium on the concentration of elements in body tissues has not been investigated yet.

Monensin is an ionophore antibiotic widely used as an anticoccidial agent in poultry and as a growth promoter in cattle (Langston et al., 1985). It has a narrow margin between therapeutic and toxic doses and improper mixing or accidental administration of higher levels usually leads to grave consequences. Therapeutic levels of selenium provide partial protection from monensin toxicosis in pigs (Van Vleet et al., 1983 and 1987) and cattle (Van Vleet et al., 1985). Malondialdehyde and GSH-Px values rose upon administration of monensin to broiler chickens (Sályi et al., 1990). Monensin at therapeutic dosages improved blood selenium and GSH-Px level in sheep and in lambs born to them (Anderson et al., 1983). However, monensin administered to sheep at therapeutic doses enhanced the toxicity of selenium and elevated the selenium levels of the tissues (Smyth et al., 1990). Effects of toxic levels of monensin upon tissue mineral concentration and selenium toxicity are not known.

The present study was undertaken to demonstrate interactions of toxic levels of monensin and selenium in broiler chicks and their effects on mortality, body weight and on the concentration of different minerals in the liver tissue.

Materials and methods

Broiler chicks (Astra B breed) kept on a balanced basal feed (BK-2 broiler grower mash, Animal Feed Production Department, Olsztynek, Poland) for two weeks were divided into nine groups of 13 birds each and their diets were amended by the addition of toxic substances as follows. Groups S0 and SM = 15 mg selenium/kg as sodium selenite (Na_2SeO_3); groups ES0 and ESM = 15 mg selenium/kg plus 200 mg vitamin E/kg as alpha tocopherol; groups M0 and MS = 240 mg monensin/kg as monensin

sodium (Elanco Laboratories, Italy). Groups 0S, 0M and 00 = control groups fed unamended basal feed.

Table 1

Plan of the experiments

a. First experiment

Groups	No. of birds	1st toxic substance in feed (mg/kg)	Adaptation period (days)	2nd toxic substance three oral doses (mg/kg body weight)
S0	13	selenium (15)	12	none
SM	13	selenium (15)	12	monensin (250)
ES0	13	selenium (15) plus vitamin E (200)	12	none
ESM	13	selenium (15) plus vitamin E (200)	12	monensin (250)
M0	13	monensin (240)	12	none
MS	13	monensin (240)	12	selenium (5)
0S	13	none	12	selenium (5)
0M	13	none	12	monensin (250)
00	13	none	12	none

b. Second experiment

The plan of the second experiment was the same as that of the first experiment with the following differences: (1) The number of birds in each group was 15; (2) The adaptation period was four weeks; (3) The second toxic chemical was administered orally on alternate days for four weeks after the adaptation period at the following dose levels; selenium 1 mg/kg and monensin 40 mg/kg body weight

Acute Exposure. After an adaptation period of 12 days on these amended feeds, acute monensin toxicosis was induced in groups SM, ESM and 0M by oral administration of 250 mg monensin/kg body weight on the first, third and fifth days. Similarly, acute selenium toxicosis was induced in groups MS and 0S by oral administration of three doses of 5 mg selenium/kg body weight. In this way the different groups were exposed to

monensin or selenium alone or to both monensin and selenium. The birds in group 00 acted as unintoxicated control. An overview of the experimental plan is presented in Table 1.

Three birds from each group were killed 24 h after exposure for the collection of internal organs. The liver of each bird was weighed, placed in a polythene bag and stored at –20 °C until used for the estimation of metal contents. The remaining 10 birds were kept under observation for up to 2 weeks to record the number of deaths in each group. Subsequently, the surviving birds in each group were killed and organs were collected and frozen.

Subacute Exposure. The plan was the same as in the first experiment except that the adaptation period was 4 weeks following which subacute toxicosis of monensin and selenium was induced by oral administration of 40 mg monensin and 1 mg selenium/kg body weight on alternate days for four weeks. The body weights of all birds in each group were recorded at weekly intervals. Three birds were sacrificed from each group in the first, second, third and fourth week post exposure and the internal organs were collected and frozen.

Estimation of metals. The liver from each bird was homogenized and a weighted portion was digested with nitric acid and sulphuric acid. Lead (Pb), iron (Fe), copper (Cu), zinc (Zn), manganese (Mn) and magnesium (Mg) concentrations were estimated by atomic absorption spectrophotometry (Pye Unicam SP 62900) (Whiteside, 1976). Selenium content was estimated by hydride generation using atomic absorption spectrophotometry (Pye Unicam SP 2900) (Brooks et al., 1983). All the values were determined on a wet weight basis.

Statistical analysis. Comparisons were made between the different groups exposed to monensin and selenium concurrently or seperately. The data were analyzed by one-way analysis of variance and group means were compared by Duncan's multiple range test at the 0.05 level of significance (Steel and Torrie, 1980).

Results

Monensin exposure. The concentrations of different elements in the liver during acute and subacute monensin toxicosis are presented in Tables 2 and 3, respectively.

Table 2

Concentration of different elements in the liver of chicks during acute monensin toxicosis (mean ± SD)

Group (dietary addition)	Monensin[1] (mg)	Se (μg/g)	Pb (μg/g)	Fe (μg/g)	Cu (μg/g)	Zn (μg/g)	Mn (μg/g)	Mg (μg/g)
				24 h post exposure				
S0 (Se)	0	3.04 ± 0.16c	0.76 ± 0.05b	131.3 ± 1.3b	2.17 ± 0.01a	20.62 ± 0.20b	1.97 ± 0.01c	99.17 ± 1.00a
SM (Se)	250	3.94 ± 0.08	0.12 ± 0.02a	163.7 ± 1.4d	2.88 ± 0.01b	19.13 ± 0.12a	2.58 ± 0.01d	94.30 ± 2.66a
ES0 (Se+E)[2]	0	4.10 ± 0.08d	0.15 ± 0.01a	76.8 ± 0.8a	3.33 ± 0.01c	27.59 ± 0.37c	0.96 ± 0.04b	290.10 ± 3.83d
ESM (Se+E)	250	3.31 ± 0.07c	1.10 ± 0.01c	150.4 ± 1.6c	3.86 ± 0.05d	37.47 ± 1.19e	0.42 ± 0.02a	149.63 ± 0.62c
0M (-)	250	1.61 ± 0.22b	0.32 ± 0.03ab	143.5 ± 0.9b	4.60 ± 0.04e	31.99 ± 0.01d	3.13 ± 0.07e	134.22 ± 0.41b
00 (-)	0	0.58 ± 0.01a	0.55 ± 0.03ab	60.4 ± 1.9a	4.58 ± 0.04e	25.23 ± 1.04c	5.76 ± 0.12f	119.8 ± 0.35b
				Two weeks post exposure				
S0 (Se)	0	3.48 ± 0.31b	0.38 ± 0.06	99.0 ± 3.23a	2.75 ± 0.15a	19.72 ± 0.45b	1.93 ± 0.17	187.85 ± 10.98b
SM (Se)	250	4.77 ± 0.22c	0.17 ± 0.02	165.30 ± 2.78c	2.91 ± 0.22a	16.46 ± 0.24a	1.71 ± 0.01	75.80 ± 0.51a
ES0 (Se+E)	0	3.87 ± 0.29b	0.26 ± 0.16	281.84 ± 7.67d	2.93 ± 0.26ab	21.10 ± 0.08c	1.36 ± 0.55	219.85 ± 8.59c
ESM (Se+E)	250	all died before the end of the 2nd week						
0M (-)	0	0.26 ± 0.03a	0.08 ± 0.02	107.84 ± 0.60b	3.61 ± 0.03c	21.13 ± 0.06c	1.72 ± 0.02	93.81 ± 0.21a
00 (-)	0	0.39 ± 0.06a	0.36 ± 0.13	89.34 ± 8.27a	3.06 ± 0.2b	22.50 ± 1.02d	1.67 ± 0.74	215.63 ± 5.84c

[1] given per kg body weight orally on the first, third and fifth day; [2] Se + vitamin E
Values in each column with different letters are statistically significant ($P < 0.05$)

Table 3

Concentration of different elements in the liver of chicks during subacute monensin toxicosis (mean ± SD)

Group (dietary addition)	Monensin[1] (mg)	Se (μg/g)	Pb (μg/g)	Fe (μg/g)	Cu (μg/g)	Zn (μg/g)	Mn (μg/g)	Mg (μg/g)
				One week post exposure				
S0 (Se)	0	4.06 ± 0.43c	0.62 ± 0.03ab	110.7 ± 22.4b	2.67 ± 0.07ab	19.13 ± 0.21a	1.93 ± 0.16	264.8 ± 12.7b
SM (Se)	40	4.71 ± 0.41d	1.35 ± 0.43b	109.7 ± 15.9b	3.64 ± 0.12c	25.34 ± 3.21b	1.92 ± 0.16	239.3 ± 17.5ab
ES0 (Se+E)[2]	0	4.97 ± 0.08d	0.38 ± 0.03a	70.1 ± 13.9a	2.21 ± 0.20a	19.89 ± 0.65a	1.58 ± 0.11	272.0 ± 18.4c
ESM (Se+E)	40	2.86 ± 0.17b	0.31 ± 0.04a	107.6 ± 14.0b	2.90 ± 0.29b	19.18 ± 2.80a	2.21 ± 0.48	219.1 ± 26.4a
0M (-)	40	0.38 ± 0.06a	0.37 ± 0.06a	147.7 ± 14.2c	4.64 ± 0.27d	18.91 ± 1.53a	2.75 ± 0.18	275.1 ± 6.2c
00 (-)	0	0.48 ± 0.01a	0.96 ± 0.38a	87.9 ± 16.0a	3.63 ± 0.68c	20.78 ± 0.64a	2.17 ± 0.42	231.1 ± 6.0ab
				Two weeks post exposure				
S0 (Se)	0	3.88 ± 0.46c	0.66 ± 0.13	101.3 ± 26.1ab	2.97 ± 0.26bc	21.68 ± 1.80ab	1.99 ± 0.14	293.2 ± 18.9bc
SM (Se)	40	2.80 ± 0.17b	0.73 ± 0.14	171.9 ± 1.7c	4.35 ± 0.83d	25.71 ± 8.10bc	2.72 ± 0.37	290.8 ± 27.1b
ES0 (Se+E)	0	2.93 ± 0.11b	0.54 ± 0.06	140.1 ± 10.1b	1.96 ± 0.13a	15.77 ± 0.84a	2.08 ± 0.04	302.3 ± 14.9c
ESM (Se+E)	40	3.37 ± 0.46c	064 ± 0.15	143.7 ± 26.0b	2.57 ± 0.14ab	21.64 ± 2.18ab	1.59 ± 0.01	289.4 ± 9.1b
0M (-)	40	0.35 ± 0.10a	0.56 ± 0.10	103.4 ± 4.0ab	3.64 ± 0.41cd	26.89 ± 1.13c	2.07 ± 0.58	264.2 ± 3.1ab
00 (-)	0	0.71 ± 0.15a	0.76 ± 0.10	73.9 ± 13.3a	3.34 ± 0.42c	21.59 ± 0.62b	2.64 ± 0.51	242.3 ± 29.1a

Table 3 continued

Group (dietary addition)	Monensin[1] (mg)	Se (μg/g)	Pb (μg/g)	Fe (μg/g)	Cu (μg/g)	Zn (μg/g)	Mn (μg/g)	Mg (μg/g)
Three weeks post exposure								
S0 (Se)	0	3.67 ± 0.51c	0.54 ± 0.07	81.6 ± 10.7a	3.13 ± 0.45ab	21.31 ± 1.88a	1.51 ± 0.14a	260.8 ± 3.3c
SM (Se)	40	2.54 ± 0.52b	0.90 ± 0.41	134.1 ± 36.4b	2.31 ± 0.32a	20.11 ± 1.54a	1.73 ± 0.03b	260.7 ± 25.7c
ES0 (Se+E)[2]	0	3.64 ± 0.61c	0.37 ± 0.07	141.3 ± 9.1b	2.74 ± 0.14a	25.85 ± 0.57b	1.84 ± 0.11b	233.5 ± 5.6b
ESM (Se+E)	40	3.12 ± 0.24bc	0.58 ± 0.11	168.2 ± 37.8b	3.03 ± 0.83ab	19.14 ± 1.07a	1.74 ± 0.11b	244.4 ± 9.2b
0M (-)	40	0.52 ± 0.09a	0.57 ± 0.12	154.1 ± 18.0b	3.27 ± 0.28a	21.73 ± 0.33a	2.23 ± 0.09c	237.5 ± 4.3b
00 (-)	0	0.17 ± 0.01a	0.29 ± 0.02	84.3 ± 13.9a	4.14 ± 0.43b	29.46 ± 2.65c	1.50 ± 0.07a	218.1 ± 10.3a
Four weeks post exposure								
S0 (Se)	0	5.29 ± 0.67d	0.59 ± 0.22	144.0 ± 25.6b	2.31 ± 0.30a	22.18 ± 0.76ab	2.09 ± 0.29b	238.8 ± 7.2
SM (Se)	40	5.47 ± 0.82d	0.42 ± 0.09	186.6 ± 30.8c	3.03 ± 0.67bc	25.25 ± 1.53b	2.20 ± 0.17b	245.4 ± 21.6
ES0 (Se+E)	0	2.89 ± 0.14b	0.44 ± 0.11	154.4 ± 8.5bc	2.16 ± 0.14a	21.94 ± 0.10a	2.66 ± 0.59b	246.2 ± 18.4
ESM (Se+E)	40	4.49 ± 0.63c	0.51 ± 0.18	158.9 ± 21.5bc	2.36 ± 0.22a	21.42 ± 0.70a	1.97 ± 0.15a	266.7 ± 18.5
0M (-)	40	0.62 ± 0.11a	0.53 ± 0.16	244.7 ± 41.2d	4.11 ± 0.14c	29.32 ± 0.49c	3.09 ± 0.17c	229.6 ± 12.1
00 (-)	0	0.51 ± 0.05a	0.50 ± 0.16	87.7 ± 4.21a	4.02 ± 0.19c	29.66 ± 1.09c	2.82 ± 0.17bc	235.8 ± 16.9

[1] given per kg body weight orally on alternate days for four weeks; [2] Se + vitamin E
Values in each column with different letters are statistically significant ($P < 0.05$)

During acute toxicosis no significant differences were found in selenium concentration in the groups fed selenium (group S0 and ES0) and those given selenium and monensin concurrently (groups SM and ESM). Birds kept on basal diet and given monensin (group 0M) had significantly higher levels of selenium at 24 h post exposure compared with control birds, but this difference was nonsignificant after two weeks. Monensin and selenium administered concurrently caused a significant increase in liver iron. Depressed copper levels in groups S0 and ES0 at 24 h and two weeks were significantly increased when monensin was administered concurrently (groups SM and ESM). Manganese concentration of the liver showed wide variations at 24 h whereas at 2 weeks the levels in different groups varied nonsignificantly.

Deaths in different groups during acute toxicosis were maximum (10/10) in group ESM and minimum (6/10) in group 0M.

During subacute toxicosis selenium levels were significantly increased at 2 and 4 weeks in the groups given monensin and selenium plus vitamin E concurrently (group ESM) compared with the group not given monensin (group ES0). Monensin significantly increased the liver iron in group 0M at all stages of the experiment. This effect was also seen when selenium was present in the diet (groups SM and ES0).

Body weights at four weeks were significantly the highest in the control group (00) followed by group 0M, and the lowest in group ESM (Fig. 1).

Selenium exposure. The concentrations of elements in the liver tissue of different groups during acute and subacute toxicosis are presented in Tables 4 and 5, respectively.

Selenium administration significantly increased the liver selenium (group 0S), but this increase was even higher at two and four weeks when monensin was concurrently administered (group MS). Liver copper significantly decreased due to the administration of selenium (groups MS and 0S) at two weeks. Zinc concentration was also significantly depressed due to selenium toxicosis at 24 h and two weeks. Magnesium level was significantly decreased and was not affected by the presence of monensin (groups MS and 0S). Deaths in different groups during acute toxicosis were the highest in group MS (6/10) followed by group 0S (4/10).

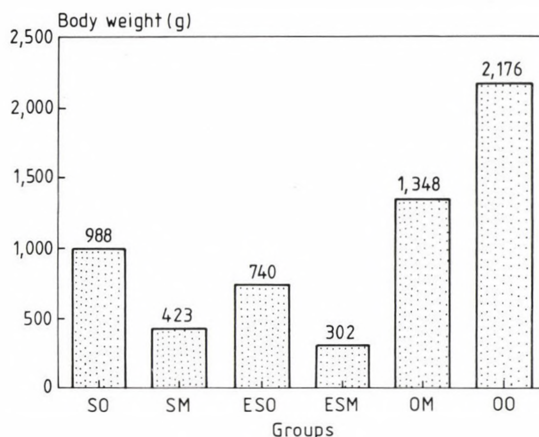

Fig. 1. Mean body weights of broiler chicks at 4 weeks post exposure during subacute monensin toxicosis and kept on a feed with or without added selenium
S0: selenium in the feed, no monensin; SM: selenium in the feed, intoxicated with monensin; ES0: selenium+vitamin E in the feed, no monensin; ESM: selenium+vitamin E in the feed, intoxicated with monensin; 0M: no selenium in the feed, intoxicated with monensin; 00: control
Values followed by different letters are statistically significant ($P < 0.05$)

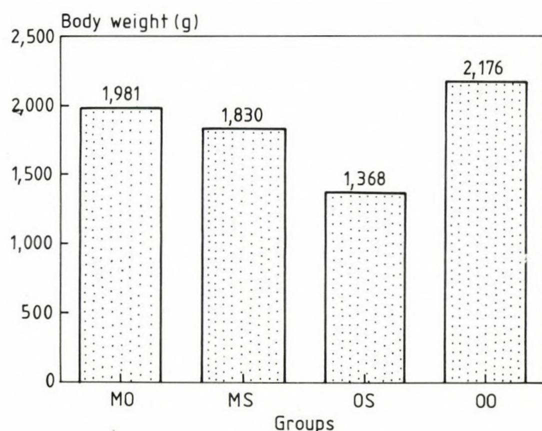

Fig. 2. Mean body weights of broiler chicks at 4 weeks post exposure during subacute selenium toxicosis and kept on a feed with or without added monensin
M0: monensin in the feed, no selenium; MS: monensin in the feed, intoxicated with selenium; 0S: no monensin in the feed, intoxicated with selenium; 00: control
Values followed by different letters are statistically significant ($P < 0.05$)

Table 4

Concentration of different elements in the liver of chicks during acute selenium toxicosis (mean ± SD)

Group (dietary addition)	Selenium[1] (mg)	Se (μg/g)	Pb (μg/g)	Fe (μg/g)	Cu (μg/g)	Zn (μg/g)	Mn (μg/g)	Mg (μg/g)
24 hours post exposure								
M0 (mon)[2]	0	0.57 ± 0.10a	0.16 ± 0.00a	46.9 ± 1.1a	4.11 ± 0.07a	22.80 ± 0.74b	2.22 ± 0.03a	75.67 ± 0.84a
MS (mon)	5	4.08 ± 0.17c	0.16 ± 0.01a	69.7 ± 0.7c	4.39 ± 0.01b	18.33 ± 0.35a	2.35 ± 0.02a	243.08 ± 15.2d
0S (-)	5	2.23 ± 0.11b	0.13 ± 0.01a	76.1 ± 2.3d	4.09 ± 0.07a	18.47 ± 0.06a	2.17 ± 0.03a	99.18 ± 1.38b
00 (-)	0	0.58 ± 0.01a	0.55 ± 0.03b	60.4 ± 1.9b	4.58 ± 0.05b	25.22 ± 1.04b	5.76 ± 0.12b	119.81 ± 0.35c
Two weeks post exposure								
M0 (mon)[2]	0	0.72 ± 0.12a	0.44 ± 0.09	86.68 ± 7.6b	3.82 ± 0.05c	28.53 ± 0.16d	2.77 ± 0.29b	264.55 ± 24.1b
MS (mon)	5	3.29 ± 0.03b	0.07 ± 0.01	99.26 ± 1.79c	1.81 ± 0.00b	17.97 ± 0.26b	2.29 ± 0.02b	114.45 ± 1.62a
0S (-)	5	2.05 ± 0.01a	0.11 ± 0.02	69.04 ± 0.70a	1.05 ± 0.02a	12.26 ± 0.09a	1.41 ± 0.06a	90.81 ± 2.44a
00 (-)	0	0.395 ± 0.06a	0.361 ± 0.13	89.34 ± 8.27b	3.06 ± 0.2c	22.50 ± 10.2c	1.67 ± 0.74a	215.63 ± 5.84b

[1] given per kg body weight orally on the first, third and fifth day; [2] monensin
Values in each column with different letters are statistically significant ($P < 0.05$)

Table 5

Concentration of different elements in the liver of chicks during subacute selenium toxicosis (mean ± SD)

Group (dietary addition)	Selenium[1] (mg)	Se (μg/g)	Pb (μg/g)	Fe (μg/g)	Cu (μg/g)	Zn (μg/g)	Mn (μg/g)	Mg (μg/g)
				One week post exposure				
M0 (mon)[2]	0	0.51 ± 0.06a	0.31 ± 0.02a	100.2 ± 24.5ab	4.07 ± 0.07b	25.90 ± 0.17d	3.41 ± 0.14b	249.4 ± 14.1
MS (mon)	1	1.95 ± 0.18c	0.40 ± 0.02a	125.3 ± 26.8b	3.35 ± 0.04a	23.31 ± 1.29c	2.70 ± 0.02ab	253.8 ± 32.2
0S (-)	1	1.41 ± 0.18b	0.67 ± 0.06ab	80.8 ± 3.5a	4.84 ± 0.24b	22.05 ± 0.79b	2.61 ± 0.60ab	237.7 ± 1.3
00 (-)	0	0.48 ± 0.01a	0.97 ± 0.38b	87.8 ± 16.0a	3.63 ± 0.68a	20.78 ± 0.64a	2.17 ± 0.42a	231.1 ± 6.0
				Two weeks post exposure				
M0 (mon)	0	0.74 ± 0.12a	0.45 ± 0.10	84.6 ± 0.1a	3.82 ± 0.06b	28.73 ± 1.21c	2.61 ± 0.37b	286.8 ± 4.1b
MS (mon)	1	1.23 ± 0.37b	0.47 ± 0.05	148.8 ± 24.8b	2.85 ± 0.53a	21.69 ± 3.68a	2.41 ± 0.24b	291.1 ± 8.8b
0S (-)	1	1.47 ± 0.30b	0.58 ± 0.05	77.5 ± 5.2a	2.63 ± 0.64a	23.08 ± 1.66b	1.86 ± 0.08a	248.5 ± 13.2a
00 (-)	0	0.61 ± 0.03a	0.75 ± 0.10	73.9 ± 13.3a	3.17 ± 0.65b	21.69 ± 0.62a	2.64 ± 0.51b	242.3 ± 29.1a

Table 4 continued

Group (dietary addition)	Selenium[1] (mg)	Se (µg/g)	Pb (µg/g)	Fe (µg/g)	Cu (µg/g)	Zn (µg/g)	Mn (µg/g)	Mg (µg/g)
				Three weeks post exposure				
M0 (mon)[2]	0	0.53 ± 0.04a	0.34 ± 0.06	138.4 ± 14.5b	4.25 ± 0.13b	24.02 ± 1.85a	1.83 ± 0.01b	228.1 ± 10.1a
MS (mon)	1	1.58 ± 0.15b	0.39 ± 0.18	126.6 ± 32.3b	3.53 ± 0.33ab	27.15 ± 3.24b	2.43 ± 0.13c	267.3 ± 9.8b
0S (-)	1	1.33 ± 0.06b	0.53 ± 0.11	111.8 ± 2.6b	3.16 ± 0.14a	24.93 ± 1.50a	1.48 ± 0.07a	253.6 ± 16.6b
00 (-)	0	0.47 ± 0.01a	0.29 ± 0.20	84.3 ± 13.9a	4.08 ± 0.53b	29.45 ± 2.65b	1.50 ± 0.07a	218.1 ± 10.3a
				Four weeks post exposure				
M0 (mon)	0	0.64 ± 0.07a	0.87 ± 0.16	164.0 ± 14.4b	3.90 ± 0.46bc	29.22 ± 2.43b	2.60 ± 0.34b	237.6 ± 17.4
MS (mon)	1	1.60 ± 0.27b	0.56 ± 0.16	150.2 ± 24.2b	3.39 ± 0.04b	27.04 ± 2.51b	2.26 ± 0.32ab	260.9 ± 17.7
0S (-)	1	1.10 ± 0.33b	0.43 ± 0.14	271.2 ± 9.5c	2.39 ± 0.22a	23.77 ± 2.65a	2.06 ± 0.11a	235.9 ± 6.9
00 (-)	0	0.51 ± 0.05a	0.49 ± 0.16	87.7 ± 4.21a	4.01 ± 0.19c	29.66 ± 1.09b	2.82 ± 0.16b	235.8 ± 16.9

[1] given per kg body weight orally on alternate days for four weeks; [2] monensin

Values in the same column with different letters are statistically significant ($P < 0.05$)

During subacute selenium toxicosis a significant increase in liver selenium concentration was observed at all stages in group 0S. The concurrent presence of monensin in the diet caused a further significant increase in selenium levels (group MS) at week 1 and 3. Selenium alone significantly raised liver iron levels (group 0S) at 4 weeks. Iron levels were significantly increased from week 1 to 4 when both selenium and monensin were administered together (group MS). The copper level of the liver was significantly depressed (group 0S) and it persisted even when monensin was present in the diet (group MS). Monensin alone did not alter the liver copper levels. Zinc level was significantly depressed by selenium administration (group 0S) at weeks 3 and 4. Manganese levels were significantly lower in birds fed selenium (group 0S) but this decrease was nonsignificant when monensin was present in the feed (group MS) at 2–4 weeks.

Body weights at four weeks were significantly lowest in group 0S as compared with the control (Fig. 2).

Discussion

The effects of monensin and selenium feeding upon different biological variables have been studied in domestic mammals (Anderson et al., 1983; Smyth et al., 1990; Langston et al., 1985). However, such information, particularly that concerning the effects upon mineral levels in the body tissues of avian species, is scarce. For this reason the results of the present experiments were compared with the findings recorded in other species given therapeutic or toxic levels of selenium and monensin.

An interaction between selenium and monensin in the present study was evidenced by the increased level of selenium in the liver tissue when both substances were administered together, as compared with the case when selenium was administered alone. Increased selenium levels of body tissues were recorded when therapeutic levels of monensin were concurrently administered to sheep during selenium toxicosis (Smyth et al., 1990). The activity of GSH-Px, a selenium-dependent enzyme, is proportionally related to the selenium levels of the body tissues (Omaye et al., 1974). An indirect evidence of the effect exerted by monensin upon body selenium level was seen when monensin administered to broiler chicks increased the GSH-Px level in the liver (Sályi et al., 1990) and erythrocytes (Khan,

1991). Similar results were reported in sheep fed therapeutic levels of monensin (Anderson et al., 1983). The higher mortality and more severe body weight depression seen during monensin toxicosis in birds concurrently fed selenium in the present study (groups SM and ESM) indicated the enhancement of toxicities. However, the nonsignificant body weight depression in chicks fed monensin during selenium toxicosis (group MS) indicated that monensin–selenium interaction does not always lead to enhanced toxicities. Selenium and vitamin E are known to modify the development of monensin toxicosis (Van Vleet et al., 1985).

Increased accumulation of lead in the liver was recorded upon its concurrent feeding with selenium (Donaldson and McGowan, 1989) and monensin (Khan et al., 1993). The nonsignificant variation in liver lead levels in the present study suggested that the reported effects of selenium and monensin might be associated only with higher dietary lead levels.

Increased liver iron due to the administration of monensin and selenium at different doses in the present study indicated that both substances affect the iron metabolism. However, the exact mechanism of these effects is not understood completely.

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THYROID HORMONE DEIODINATION IN THE BRAIN OF YOUNG CHICKENS ACUTELY ADAPTS TO CHANGES IN THYROID STATUS*

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The brain represents a special organ in respect of thyroid hormone handling. This was analyzed in one-week-old chickens. The effect of sham-operation (SH), thyroidectomy (TX) and thyroidectomy plus thyroxine supplementation (TX+T4) on the intracerebral triiodothyronine (T3) content and on the activity of different types of deiodinases was investigated. it was found that in spite of very low T3 levels in the serum of TX animals, the brain displayed close to normal tissue T3 levels. Kinetic studies of the deiodinase system showed an increase in type II activity (increased T4 to T3 conversion) and decreased type III activity (decreased degradation of intracellular T3) in the brain of TX animals vs. SH or TX+T4. It is concluded that a considerable part, if not the total of the T3 preserved in hypothyroidism may be ascribed to adaptive changes of the deiodinase system in the brain of young chickens.

Key words: Brain, chicken, deiodination, thyroid metabolism

The normal development and adequate function of the central nervous system requires intact thyroid machinery, both in the human (Eayrs, 1960) and in animals (Balázs et al., 1986, 1969). The damages imposed on normal brain development by hypothyroidism at some critical time periods are irrevocable, and cannot be corrected by delayed treatment with thyroid hormones (Ford and Cramer, 1977). Most probably this is the reason for the fact that a unique, yet not fully understood mechanism exists in the brain that is capable of maintaining intracerebral thyroid hormone concentrations nearly at normal level in mammals, even if the concentration of serum thyroid hormones is low (Silva and Larsen, 1982; Obregon et al., 1986; Ruiz de Ona et al., 1988). A similar mechanism was found in chickens (Rudas 1988*a,b*; 1989) We have shown that the concentration of the

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active thyroid hormone, triiodothyronine, in the brain of young chickens remains close to normal even after 7 days of thyroidectomy in spite of the almost undetectable serum T3 concentration, and relatively low tissue concentrations in other organs at the same time (Rudas and Pethes, 1989). It was concluded that at least three separate mechanisms could exist that may be responsible for the above phenomenon. Changes of the membrane-dependent uptake and elimination of the active thyroid hormone (T3) and/or that of the precursor hormone, thyroxine (T4) are the first two possibilities. Indeed, it could be shown that the uptake of T4 and T3 is tremendously increased by the brain of hypothyroid chickens versus euthyroid ones (Rudas, 1989). The third possibility besides uptake and elimination, which may explain such adaptation is the appropriate alteration of the activity of the deiodination enzyme complex that governs the peripheral metabolism of thyroid hormones in both mammals (Kaplan, 1984*a*) and chickens (Kühn et al., 1984; McNabb, 1988). This paper gives an analysis of the latter possibility, namely it investigates the behaviour of chicken brain deiodinases during hypothyroidism using enzyme kinetic methods in order to describe them according to the latest classification (Leonard and Visser, 1986; Berry and Larsen, 1992) of this unique class of enzymes.

Experimental animals

Hunniahybrid broiler chickens were used throughout this study. One-week-old birds were obtained from a commercial broiler farm (Ócsa Coop., Budapest) and were raised in the animal house of our laboratory. Food and water was available *ad libitum*, and a continuous lighting (24L:00D) regime was applied.

Thyroidectomy

Surgical thyroidectomy was carried out at two weeks of age under Nembutal anaesthesia (25 mg/kg, i.v. in 100 μl saline/100 g bwt) according to our previously described method (Rudas and Pethes, 1986). The effectiveness of thyroidectomy was checked by measuring the concentrations of T4 and T3 in the serum.

T4 and T3 plasma concentrations

A radioimmunoassay developed in our laboratory earlier (Pethes et al., 1978) was used to measure T4 and T3 level in both serum and tissue extracts. The interassay variance of this assay is less then 10 per cent, while the intraassay variance is 5–7 per cent.

Tissue concentrations of T3

Deep-frozen brain samples were allowed to thaw at room temperature for not more than 5 min. The tissue was then homogenized in 0.15 M phosphate buffer (2 ml for one gram of tissue), pH 7.4 containing 100 mM propylthiouracyl to block *in vitro* deiodination. This homogenate was then extracted according to Nejad et al. (1975). Alcoholic extracts resulting from this procedure were kept under –20 °C until T3 RIA was performed. 131-I-T3 (spec. act. >1200 Ci/g; Izinta, Budapest) was used to check the efficiency of extraction, which ranged between 85–90 per cent. The concentration of T3 obtained by this procedure was also checked by another approach described earlier (Rudas and Pethes, 1989). 0.5 μCi 131-I-T3/animal was injected intravenously and the tissue to serum ratio (T/S) of this hormone was determined at equilibrium time point after paper chromatography of the tissue. The tissue concentration of T3 was calculated using this ratio and the serum T3 concentration from parallel RIA measurements. The amount of plasma contaminating the brain was taken into account as follows. 131-I-albumin (labelled by the chloramin-T method) was prepared. In a pilot study 5 TX and 5 SH chickens were injected with 5 μCi of this label 24 h after sugery. One, 2, 5 and 10 min thereafter blood samples were collected, and at the last blood sampling animals were decapitated, the brain removed and the amount of plasma per wet tissue weight determined using the tissue to plasma ratio of albumin. The result was 13 ± 2 μl in TX and 12.5 ± 2.4 μl plasma per gram of tissue. This figure was used in corrections made for tissue T3 determinations.

Deiodinase assay

Deiodinase measurements were done according to the methods of Silva et al. (1982) as strictly as possible. Briefly, this entailed the followings.

Method 1: Activity measurements. Crude homogenates of brain tissue were made in phosphate buffer (3:1 vol/w; 0.15 M, pH 7.5) using an Elvehjem-Potter type homogenizer (10 sec, at fok Crude homogenates of brain tissue were made in phosphate buffer (3:1 vol/w; 0.15 M, pH 7.5) using an Elvehjem-Potter type homogenizer (10 sec, at 4 °C). The three types of deiodinases were measured separately, using different conditions. In all three cases 100 µl of the brain homogenate was used and an additional 100 µl of the same buffer was added with the following substances: 40 µl 125-I-labelled iodothyronine (as indicated later) the activity of which was adjusted so that 10–25 per cent of it was used up by the enzyme in a 30-min incubation period; 30 µl dithiothreitol (DTT) as a cofactor; 30 µl propylthiouracyl (PTU) as an inhibitor. DTT and PTU were used in different concentrations (see later). Tubes with or without cofactor, inhibitor and homogenate were incubated simultaneously at 37 °C for 30 min. The reaction was then stopped using 1 ml ice-cold ethanol containing 100 mM PTU. This was followed by centrifugation (3,000 *g*, 20 min, 4 °C). The labelled iodine present in the supernatant was quantitated by paper chromatography. Results were expressed as pmol or fmol iodine released/min/mg protein. Protein concentration was measured according to Lowry et al. (1951). The specific conditions for different types of deiodinases were as follows. Type I: 1.5 µM 125-I-reverse-T3 (spec. act. >1,200 Ci/g; Izinta, Budapest) was used as substrate. DTT concentration was 8 mM (Leonard and Rosenberg, 1980). Type II: 125-I-T4 was used as substrate (2 nM) in the presence of 25 mM DTT and 1 mM PTU. Type III: 1.5 nM 125-I-T3 was used as substrate, in the presence of 80 mM DTT, and 1 mM PTU. Fresh labels were used throughout, and parallel tubes were incubated for checking the impurity of the label which, as a rule, was less than 2%.

Method 2: Kinetic study. Based on the above method the maximal velocities (Vmax) and the Michaelis-Menten constants (Km) for the three types of deiodinases were determined. In all three cases this was done by introducing increasing amounts (five points) of the respective non-labelled iodothyronine (T3 and T4 was purchased from Sigma Chem Co., St.

Louis, USA; r-T3 was obtained from Henning GmbH, Germany) into the incubation medium. The concentrations of non-labelled iodothyronines were adjusted in pilot experiments so as to be close to the expected Km of the enzyme. The reciprocal of product formation velocity was then plotted versus the reciprocal of the subtrate concentration (Lineweaver-Burk diagram), from which the appropriate kinetic parameters were obtained both, visually and by regression equations (Enzfitter programme, Jandel Corp., U.S.A.). In pilot studies product formation was linearly proportional to the protein concentration of the homogenate.

Chromatography

The tertiary amyl alcohol/hexane/ammonia descending paper chromatography system was used as originally described by Bellabarba et al. (1968) in the case of the enzyme studies. The same method was used while determining tissue concentrations of T3 with the modifications suggested by Silva and Matthews (1984).

Experiments

Trial 1: T3 content of brain tissue. Two-week-old chickens were used. Fourteen animals were thyroidectomized (TX) and 14 birds were sham-operated (SH). Both groups were kept under identical conditions and allotted to two subgroups. Subgroups labelled IOP(–) were non-treated, subgroups labelled IOP(+) were treated with 10 mg iopanoic acid (IOP) ip. 6 and 18 h after operation. Groups of 7 chickens after Tx were supplemented with T4 according to a schedule that was successful formerly (Kühn et al., 1984) to achieve physiological T3 serum concentrations in thyroidectomized chickens (group TX+T4). The T4 dose was 2 μg/kg/day in two aliquots, 6 and 18 h after surgery. This dose equals the normal daily production rate. Twenty-four h after surgery the animals were injected with 0.5 μCi 131-I-T3/animal. Three h later (at equilibrium time point for T3, as found earlier) the birds were sacrificed. Blood samples were collected and the brain was removed after dissecring the skull. The brain was placed on a filter paper, cleaned carefully from the dura mater so that all the vessels on the surface and on the basis of the cerebrum were removed. The brain without cerebellum and brain stem was severed from the rest of the brain

and it was then kept frozen at –20 °C until determinations. 131-I-T3 activities were determined immediately following the experiment. RIA measurements were made 2 months later (at a time when 131-I activity was negligible) when T4 and T3 content of the plasma and the T3 content of the brain tissue were determined.

Trial 2. Thyroidectomy (TX) and sham (SH) operations were performed on 21 seven-day-old chickens. T4 supplementation was done as in trial 1. Twenty-four h after the operation groups of SH, TX and TX+T4 chickens were bled by cervical dislocation. The plasma was separated and kept frozen together with the brain samples that were dissected out and carefully cleaned from dura and vessels (see above). The activity and kinetic parameters of the different deiodinases were determined after storing the tissues under –20 °C for 2 months.

Results

The concentration of T4 and of T3 in the serum of thyroidectomized and of sham-operated animals during trials 1 and 2 are given in Table 1. It can be seen that TX animals have very low serum levels after thyroidectomy in both trials. IOP treatment in trial 1 significantly decreased T3 levels, and caused a slight increase in T4 serum levels.

The concentrations of tissue T3 are demonstrated in Table 2. It is found that thyroidectomy depressed brain levels of triiodothyronine, however, this depression is far less than that found in the serum. A non-significant drop of about 10 per cent is seen. In IOP-treated SH animals, the T3 content of the brain is depressed by about 50 per cent of the original (SH, IOP–) concentration ($P<0.01$). In IOP treated plus thyroidectomized birds the tissue level of T3 was about one third of that found in the sham-operated IOP-treated group ($P<0.01$). T4 supplementation was unable to restore pre-operation levels of T3 in the brain of thyroidectomized plus IOP treated animals ($P<0.01$).

Table 1

Serum thyroid hormone concentrations in surgically thyroidectomized (TX), sham-operated (SH) or thyroidectomized plus T4 supplemented (TX+T4) groups of chickens in trials 1 and 2 [nmol/L±SEM(N)]

			T4	T3	T3 in % of SH, IOP(–)
Trial 1	IOP (–)	SH	12.5 ± 1.2(7)^{A}	3.55 ± 0.45(7)^{A}	100.0
		TX	0.87 ± 0.11(7)^{A}	0.11 ± 0.04(7)^{A}	3.1
		TX+T4	16.7 ± 1.87(7)	4.32 ± 0.55(7)	122.0
	IOP (+)	SH	18.9 ± 1.8(7)^{A}	0.45 ± 0.11(7)^{AB}	12.7
		TX	1.26 ± 0.14(7)	ND	-
		TX+T4	28.2 ± 2.15(7)^{A}	0.10 ± 0.04(7)^{B}	2.8
Trial 2		SH	15.15 ± 2.8(7)^{B}	3.27 ± 0.14(7)^{C}	
		TX	1.45 ± 0.11(7)^{B}	0.22 ± 0.05(7)^{C}	
		TX+T4	18.21 ± 1.87(7)	4.23 ± 0.37(7)	

Groups with same superscript in the column differ significantly (at least $P < 0.05$); ND = not detectable

Table 2

Concentration of T3 in the brain tissue of sham-operated (SH), thyroidectomized (TX) or thyroidectomized plus T4 supplemented (TX+T4) groups of chickens in trial 1, 24 + 3 h after operation as measured by RIA [nmol T3/g tissue ± SEM(N)]

			T3	T3 in % of SH, IOP(–)
Trial 1	IOP (–)	SH	1.85 ± 0.21(7)^{ABCD}	100.0
		TX	1.67 ± 0.11(7)	90.2
		TX+T4	2.12 ± 0.23(6)	114.5
	IOP (+)	SH	0.98 ± 0.18(7)^{AD}	52.9
		TX	0.29 ± 0.10(7)^{BD}	15.7
		TX+T4	0.18 ± 0.07(7)^{C}	9.7

Calculated results for tissue T3 content (based on 131-I-T3 tissue to serum ratio and serum T3 content) were not significantly different from the given values. Same superscripts in the same column represent significant differences at least at the level of $P < 0.05$

Results on deiodinase activities in the brain tissue can be found in Table 3. The Km values of the enzymes did not change either in thyroidectomized or in thyroidectomized plus T4 supplemented groups when compared to sham-operated animals. There was a specific difference in the response to thyroidectomy among the three types of deiodinases as for their maximal product formation velocities (Vmax). The latter did not change in type I enzyme, but was very much increased upon thyroidectomy in the case of type II enzyme. In contrast to this finding, the Vmax of type III deiodinase was significantly decreased in TX animals. The changes produced by thyroidectomy in this respect could be restored to normal by supplementing Tx animals with T4.

Table 3

Kinetic parameters (Vmax, Km) of type I, II and III deiodinases in the brain of sham-operated (SH), thyroidectomized (TX), and thyroidectomized plus T4 supplemented (TX+T4) chickens 24 h after operation

		Type I		Type II		Type III	
		Vmax	Km	Vmax	Km	Vmax	Km
SH							
	mean	1.32	4.90	17.5^{B}	2.15	61.2^{A}	150
	± SEM	0.31	0.11	1.8	0.62	9.8	20
TX							
	mean	1.21	4.50	153.6	1.54	22.3	165
	± SEM	0.20	0.14	14.1	0.34	6.9	12
TX+T4							
	mean	1.41	3.95	22.8^{B}	2.24	68.8^{A}	168
	± SEM	0.28	0.22	6.2	0.41	7.8	19

Vmax = fmol I/mg/h; Km: nM for type I and II, µM for type I; n = 7; groups with the same superscript in the same column are statistically different from TX: $^{A}P < 0.05$, $^{B}P < 0.001$

Discussion

Serum values of thyroidectomized birds were very low in both trials, meaning that the animals were hypothyroid, and not athyreotic at least in terms of the plasma levels of thyroid hormones. The level of thyroid hormones in TX groups in both trials, when IOP was not applied, does not drop to or below detections limits. This agrees with the fact that the time elapsed after thyroidectomy is only about three times the half life of T4 and T3 in chicken (May et al., 1974; Rudas and Pethes, 1984). Thus the low, but not undetectable serum thyroid hormone levels do not reflect the imperfection of the thyroidectomy. The latter can only be judged a week after surgical thyroidectomy (Rudas and Pethes, 1989), when at least 10 times the half life has elapsed. T4 supplementation was able to restore plasma levels of thyroid hormones to, or slightly above euthyroid levels, which is in agreement with our earlier findings (Kühn et al., 1984). The application of IOP, a radiocystographic agent known to block all types of deiodination (Wu et al., 1978), further depressed T3 serum levels in TX animals, and caused a significant increase of T4 serum levels in T4-supplemented TX birds where T3 levels were not restored to normal at the same time. These effects further support the well-documented deiodination blocking and T4 preserving effect of IOP in birds that was shown by several laboratories and also by us (Decuypere et al., 1981; Rudas, 1986). There are few data on the tissue levels of thyroid hormones in the brain of birds *in vivo* (Rudas and Pethes, 1989) and in this way it is hard to contrast the established values obtained by us to results from other laboratories. The range of the tissue concentration (around 2 nmol/g tissue) is a little bit lower, but still in the range of tissue T3 levels found in other species e.g. the rat (Silva and Matthews, 1984). More important in respect of validation of the given data is the fact that we applied two methods (direct measurement of tissue T3 content by RIA and calculation of the tissue T3 level using serum T3 concentrations plus tissue to serum ratio of radiolabelled tracer T3). These two methods yielded closely agreeing results in each experimental group in trial 1.

The tissue level of the active thyroid hormone (T3) is in striking contrast with the serum levels in hypothyroid animals. Namely, the level of T3 in the brain does not drop down to 3.1% as serum level does, but remains about 90% of the original tissue level. At the same time the blocking

of peripheral deiodination with IOP leads to undetectable serum levels, but not to undetectable tissue levels of T3. A possible explanation for this might be that the blood-brain barrier prevents IOP from getting into the site in the brain where deiodination takes place. This is however highly unlikely according to our former study (Rudas and Pethes, 1989), where we could observe the same degree of inhibition of brain and liver deiodination by IOP *in vitro*. A more plausible reason for this apparent inability of IOP to depress intracerebral T3 production to zero is that other mechanisms, like an increase in T3 plasma-to-tissue transfer and a decrease of T3 tissue-to-plasma clearance rate are compensating the IOP effect in hypothyroid brain tissue. In fact such kinetic changes have been shown for rats (Silva and Matthews, 1984) and also for chickens (Rudas, 1989).

Few data are available on the characterization of different types of deiodinases in birds. Borges et al. (1980), Rudas (1986), McNabb (1988) and Darras (1992) have made some attempts to compare the characteristics of deiodinases in birds to the standard classification originally given for the rat. This paper suggests that the basic types of deiodinases in chicken brain do not differ basically from that found in the mammalian brain, if similar conditions are applied. Namely, with the widely accepted PTU sensitivity test we could justify the presence of all three types of deiodinases, with similar kinetic characteristics as found in the rat (Kaplan, 1984*b*; Leonard and Visser, 1986).

Thyroidectomy did not change the activity or the Km of type I deiodinase in the brain, which is in agreement with our earlier finding (Rudas and Pethes, 1989). This enzyme is known to respond to hypothyroidism with a decreased activity in the liver and in the kidney (Kaplan and Utiger, 1978; Rudas and Pethes, 1989). At the same time there was a nine times increase in the activity of T3 generation from T4 by the increasing activity of type II deiodinase. This increase itself may partly explain the close to normal tissue T3 concentrations found here and earlier (Rudas and Pethes, 1989) in the brain of hypothyroid chickens and it is in good agreement with data published by Silva and Matthews (1984) and Silva and Larsen (1982), who showed that type II deiodination may exhibit a compensatory increase when availability of the precursor hormone (T4) is diminished from the serum.

An additional fact contributing to preserving tissue T3 levels may be the decreased activity of type III deiodinase found here. This enzyme is

known to be present in the brain, placenta and pituitary in mammals (Kaplan, 1984*a*; Kaplan and Yaskoski, 1980) and now it is being demonstrated in the brain of chicken. Type III deiodinase degrades intracellular T3, and thus increases the elimination of this hormone in normal animals. However, in hypothyroidism it is likely that the decrease of its activity contributes to the maintenance of close to normal active thyroid hormone (namely, T3) levels.

One can conclude that the decreased availability of thyroid hormones from the serum in the brain of hypothyroid chickens will not lead to the expected depletion of this organ of thyroid hormones.This is mainly due to the compensatory mechanism of peripheral deiodination of iodothyronines in the brain of the chickens. It is seen however that other autoregulatory changes may be involved in preventing the young animals' brain to become hypothyroid at the tissue level, namely hormone uptake and elimination characteristics might also be changed in hypothyroidism.

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THYROXINE AND TRIIODOTHYRONINE UPTAKE BY THE BRAIN OF CHICKENS*

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The instant uptake of 125-I-labelled triiodothyronine and 125-I-thyroxine by the brain tissue was measured in sham-operated (SH) controls, thyroidectomized (TX) and thyroidectomized plus iodothyronine supplemented (TX+T3 or TX+T4) young chickens. It was found that thyroidectomy significantly increases the uptake of iodothyronines, and iodothyronine supplementation returns the uptake close to sham-operated levels. The characteristics of the inhibition of the uptake of labelled hormones by non-labelled iodothyronines speak in favour of the presence of specific, separate uptake sites for T3 and T4 in the blood–brain barrier of young chickens.

Key words: Brain, chicken, deiodination, thyroid hormones, thyroxine, triiodothyronine

The biological activity of thyroid hormones is determined by the occupancy of nuclear receptors in the brain (Larsen et al., 1981; Silva and Larsen, 1986). Before triiodothyronine or thyroxine gets into these intracellular sites, they should be taken up from the plasma by the cell itself, and therefore the blood–brain barrier (Bb) must play a very important indirect effect on the availability of thyroid hormones for the brain nuclei *in vivo.* It is also presumed that the Bb represents a regulatory site of hormone uptake (Krenning et al., 1983) together with the hormone-transporting proteins in the plasma (Robbins and Rall, 1960; Ekins, 1986; Terasaki and Pardridge, 1987). It was seen in rats (Silva and Matthews, 1984*a*, 1984*b*) and in chickens (Rudas, 1986; Rudas and Pethes, 1989) that in hypothyroidism the brain may exhibit a considerable autoregulation and may avoid cellular hypothyroidism. In chickens e.g. T3 generating pathways of deiodination and tissue clearance from the brain cells of triiodothyronine are altered in a way that preserves T3 in the cells (Rudas, 1988). Knowing

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the above facts it was reasonable to suppose that besides elimination and intracellular deiodination the uptake of thyroid hormones into brain cells may be a factor that is actively regulated in hypothyroidism to avoid dangerously low intracellular thyroid hormone levels of the brain in a hypothyroid environment. In the following experiments we attempted to reveal the extent to which the uptake of triiodothyronine and thyroxine into the brain is influenced by hypothyroidism.

Materials and methods

Birds

One-week-old Hunniahibrid chickens, purchased from a commercial source (Vörös Október TSz, Ócsa), were used throughout. Birds arrived at the laboratory just after hatching. Room temperature, water and food supply were regulated according to the guidelines of the supplier. On the day of arrival the birds were randomly allotted to smaller groups (16–24) of similar weight. All experiments were carried out with individually labelled chickens. Although 5 birds belonged to one treatment group, 8 chickens were prepared, since the injection technique (see later) required exact short-term timing and some animals had to be exluded from the final evaluation.

Thyroidectomy. Surgical thyroidectomy was carried out under Nembutal (25 mg/kg body mass) anaesthesia at 3 days of age. Both the right and the left glands were removed without damaging the airsacs. Thyroidectomy was considered to be complete when both the right and the left organs could be removed in their unhurt capsule (Rudas and Pethes, 1989; Veresegyházy et al., 1986).

Bolus injection technique. The uptake of iodothyronines was determined using the Oldendorff technique of single pass rapid injection (Pardridge, 1981; Pardridge and Landaw, 1987). 100 μl saline (with 0.02 g/L albumin to prevent labelled hormones to stick on syringe and cannulae, or chicken serum containing the labelled hormones (125-I-triiodothyronine, 125-I-thyroxine, 5 μCi, specific activity >1,200 μCi/g). This bolus was injected as rapidly as possible (2 sec) into the left internal carotid artery of the anaesthetized chickens. After 10 seconds the birds were decapitated

and the head was snap-frozen in dry ice–aceton within 2 sec. Extreme care was taken to maintain time: those cases where time could not be kept were discarded from further processing. The concentration of the iodothyronine in the tissue was determined after chromatography as described elsewhere (Rudas and Pethes, 1989). The uptake was calculated as this concentration (% of dose/g tissue/100 g bwt) minus the fraction of the injected iodothyronines that were not taken up by the cells but remained in the capillary bed of the tissue while the bolus had passed the system. The latter fraction was determined as follows. Groups of sham-operated or thyroidectomized chickens were injected the same way as above but with a 100 μl bolus of 5 μCi 125-I-albumin and not iodothyronine. Exactly 10 sec later the remaining concentration of labelled albumin in the different parts of the brain was determined. It was found that 1.92 ± 0.11 and 1.87 ± 0.14 (average ± SEM) % of dose/g brain/100 g bwt of 125-I-albumin was retained after this period of time in sham-operated and thyroidectomized birds, respectively. Accordingly, 1.9 per cent was subtracted from the % of dose/g tissue/100 g bwt iodothyronine concentrations to obtain the uptake.

In pilot experiments evidence was obtained that the cannula reached the desired vessel, that is the a. carotis interna. This was done in two ways. First, the route of a bolus of Iopamiro-370 (an angiographic contrast agent) was followed under X-ray. This method also proved the lack of capillary injury during rapid injection. Second, vessels of the neck and brain area were made visible post mortem in animals previously injected with liquid caoutchouc.

Experiments

Experiment 1. Instant uptake of iodothyronines by the brain. Single-pass, Oldendorff-type technique was used to determine the uptake of 125-I-T3 or that of 125-I-T4 by different parts of the brain of sham-operated control (SH), surgically thyroidectomized (TX) and surgically thyroidectomized + triiodothyronine supplemented (TX+T3) groups of two-week-old chickens. The 100 μl bolus injected into the internal cerebal artery contained the radioactively labelled tracer (5 μCi), saline and trace amounts of bovine serum albumin (0.02 g/L) to avoid sticking of the iodothyronines onto the surface of the syringe or tubings. This amount of albumin has very little effect on the diffusion characteristics of the tracers (Whitten and Fer-

guson, 1990). Five chickens were included in each group. Exactly 10 sec after the injection of the tracers the concentration of the tracers was measured in the hyperstriatum (HS), the brain stem (BS) and the cerebellum (CM) and was expressed as per cent of injected dose per gram tissue, normalized to a bird weighing 100 g (% dose/gr tissue/100 g bwt) and corrected for the remaining fraction as described before under "Bolus injection technique".

Experiment 2. Instant appearance of iodothyronines in the jugular vein. Groups, group size, tracers, bolus size, bolus content and site of injection were the same as in Experiment 1. Sampling was different. Samples were taken through a cannula inserted into the vena jugularis. Immediately after the rapid injection of the tracer into the a. carotis interna, blood samples were taken from the jugular vein in 4-sec intervals through 60 sec (15 samples). In a pilot experiment, the rate of blood flow of SH, TX and TX+T3 chickens was measured directly: no significant differences were found.

Experiment 3. Specificity of uptake sites. Sham-operated control (SH), and surgically thyroidectomized (TX) animals were used. The bolus injected was manipulated as follows. Increasing amounts of unlabelled thyroxine (0, 2.57, 7.97, 23.16 nmol/L) or unlabelled triiodothyronine (0, 0.66, 1.99, 9.29 nmol/L) and trace amounts of 125-I-T3 or 125-I-T4 (5 μ Ci) were given to chicken serum and then 100 μl was injected into the a. carotis interna as a rapid, single bolus. Sampling, and calculation of the tissue uptake of the labelled hormones were made as described before under Experiment 1.

Results

Results obtained on the uptake of labelled iodothyronines in Experiment 1 are shown in Fig. 1. Thyroidectomized groups exhibited a significantly ($P<0.01$) higher uptake of both 125-I-T3 and 125-I-T4 than sham-operated controls or T3-supplemented animals. This was true for all parts of the brain investigated. T4 was taken up into the HS to a significantly greater extent than triiodothyronine. There was a significant difference in the uptake of the different parts of the brain. HS seems to be the most active, while cerebellum the least active site of uptake for both iodothyronines.

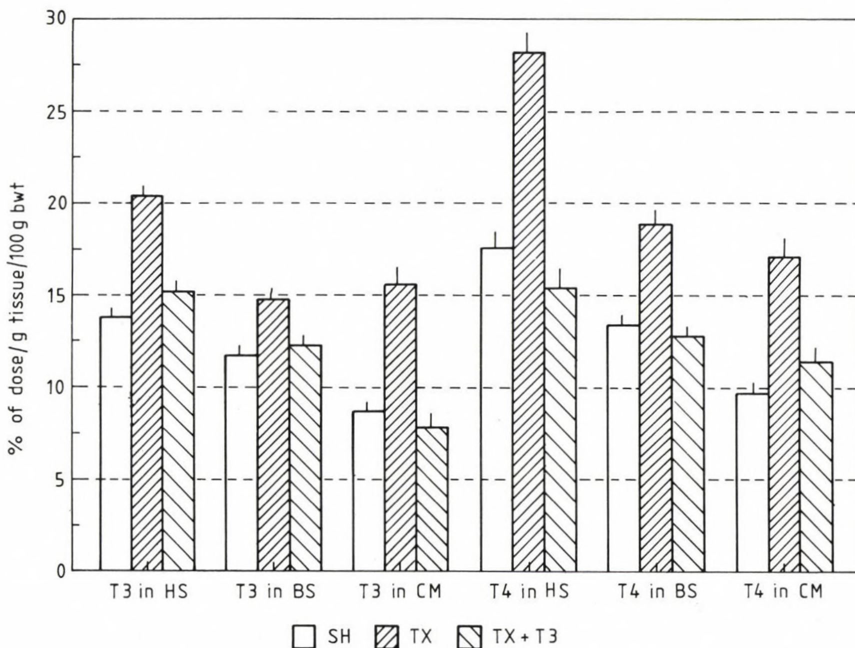

Fig. 1. The concentration of labelled triiodothyronine (T3) or labelled thyroxine (T4) in the hyperstriatum (HS), brain stem (BS) and cerebellum (CM) of sham-operated control (SH), surgically thyroidectomized (TX) and thyroidectomized plus triiodothyronine supplemented (TX+T3) chickens after a rapid (1 sec) bolus of the tracer into the internal carotid artery 10 sec after the injection. Mean ± SEM (n=5). $P<0.01$ for TX vs. SH (or TX+T3) in all parts of the brain; $P<0.01$ for T3 or T4 in the HS vs. T3 or T4 in the BS or CM; $P<0.01$ for T4 in HS vs. T3 in HS

In Experiment 2 the highest concentrations of labelled T4 or T3 appeared in the jugular vein of sham-operated controls (SH) and T3-supplemented thyroidectomized animals (TX+T3), while the TX groups exhibited relatively low peak concentrations of labelled iodothyronines in the jugular vein. (Fig. 2, panel A: uptake of labelled T3; panel B: uptake of labelled T4). The integrated area under the curves (% of dose/g tissue/100 g bwt x time, 4–60 sec) was compared by Student's *t* test. The difference was significant for both iodothyronines (TX vs. SH: $P<0.001$).

Table 1

Effects of cold triiodothyronine or thyroxine upon the uptake of 125-I-T3 and 125-I-T4 by the hyperstriatum (HS), brain stem (BS) and cerebellum (CM) of sham-operated control (SH) and surgically thyroidectomized (TX) chickens (n=5)

Groups	Bolus content		Parts of the brain investigated				
		HL		BS		CM	
		Max.	UD_{50}	Max.	UD_{50}	Max.	UD_{50}
Controls							
	125-I-T3+T3						
	mean	9.76*	14.8	7.86*	11.2	6.87*	15.4
	± SEM	0.84		1.11		0.89	
	125-I-T3+T4						
	mean	8.82	42.9	8.12*	28.9	7.13*	36.1
	± SEM	0.98		0.97		0.81	
	125-I-T4+T4						
	mean	13.12*	15.2	9.63*	12.4	6.12*	11.6
	± SEM	1.26		1.14		0.78	
	125-I-T4+T3						
	mean	12.59*	20.2	7.87*	21.3	5.81*	19.2
	± SEM	1.05		0.88		0.62	
Thyroidectomized chickens							
	125-I-T3+T3						
	mean	13.94	10.4	11.60	12.1	9.96	14.1
	± SEM	0.83		0.97		0.56	
	125-I-T3+T4						
	mean	11.96	38.5	13.70	65.6	14.60	39.7
	± SEM	0.75		1.14		1.04	
	125-I-T4+T4						
	mean	26.31	19.1	15.30	18.2	12.20	16.5
	± SEM	1.63		1.14		0.87	
	125-I-T4+T3						
	mean	21.55	28.1	13.90	37.5	14.30	41.4
	± SEM	1.68		1.09		1.14	

Max. = maximum binding of the labelled iodithyronine in the absence of cold iodothyronine (% of dose/g tissue/100 g bwt); UD_{50} = dose of cold hormone at which inhibition is 50% of the maximal uptake, nmol/L). Mean ± SEM (n=5.) (* $P<0.05$, sham-operated control group vs. the respective thyroidectomized group in the same column)

The characteristics of the uptake of labelled T3 and labelled T4 in the presence of increasing amounts of unlabelled T3 or T4 in the hyperstriatum (HS) of TX and SH chickens is presented in Fig. 3. Cold hormones inhibited the uptake of the identical labelled hormones. Non-identical cold hormones caused a 1.6–3.9 times less inhibition. In this respect TX and SH groups behaved similarly. The area under the curve (% of dose/g tissue/100 g bwt x nmol/L) in TX animals was significantly higher than in SH ones for both thyroxine and triiodothyronine. The inhibitory abilities of uptake were expressed as the dose of cold hormone required for the 50 per cent inhibition of the uptake, UD_{50}. This was calculated by a ligand binding software (Enzfitter, Elsevier-Biosoft, New York) on the basis of the theoretical considerations on ligand binding by Ross and Gilman (1985). The correlation coefficient of the regression analysis for UD_{50} was greater than 0.961 when the same cold hormone was added as the labelled hormone, and above 0.945 for non-identical label-cold hormone pairs. Since results were similar in all parts of the brain investigated, data on brain stem (BS) and cerebellum (CM) are not given graphically. The initial uptake at 0 concentration of the cold hormone and the UD_{50} values obtained are given in Table 1.

Discussion

The uptake of thyroid hormones into the brain is an important step before the establishment of contact between the hormone and its nuclear receptor (Larsen et al., 1981; Krenning et al., 1978). On the basis of their lipophilicity, thyroid hormones were supposed to enter into the cells via passive diffusion (Lein and Dowben, 1961). Later several authors proved the presence of active, energy-dependent uptake sites (Krenning et al., 1978; Halpern and Hinkle, 1982) for thyroid hormones.

The Oldendorff technique used herein presumes that the rapidly injected solution behaves like a single bolus while passing through the anatomical room investigated (Pardridge, 1981). Several authors (Ekins, 1986; Whitten and Ferguson, 1990) have questioned the feasibility of the Oldendorff technique since a dilution of the injected bolus by the following plasma may occur. To avoid this we made an indirect attempt to select optimum time for the beginning of the sampling in Experiment 2. Here the concentration of the iodothyronines in the effluent blood was determined.

It was found that peak concentrations occur 12–15 seconds after injection of the bolus (Fig. 2). Using 10 sec in our experiment guaranteed with high certainty that only very little dilution of the bolus could have occurred. Also, the fraction of the bolus remaining in the capillary bed after the single-pass may cause an error in estimating the uptake of hormone by the cells. Therefore the fraction remaining after 10 sec was experimentally determined, and the iodothyronine concentrations were corrected with this fraction. Furthermore, results depend on how precisely one can keep time in these experiments. Therefore, more animals were prepared than statistically needed and only those cases were included in the evaluation where time was exactly kept.

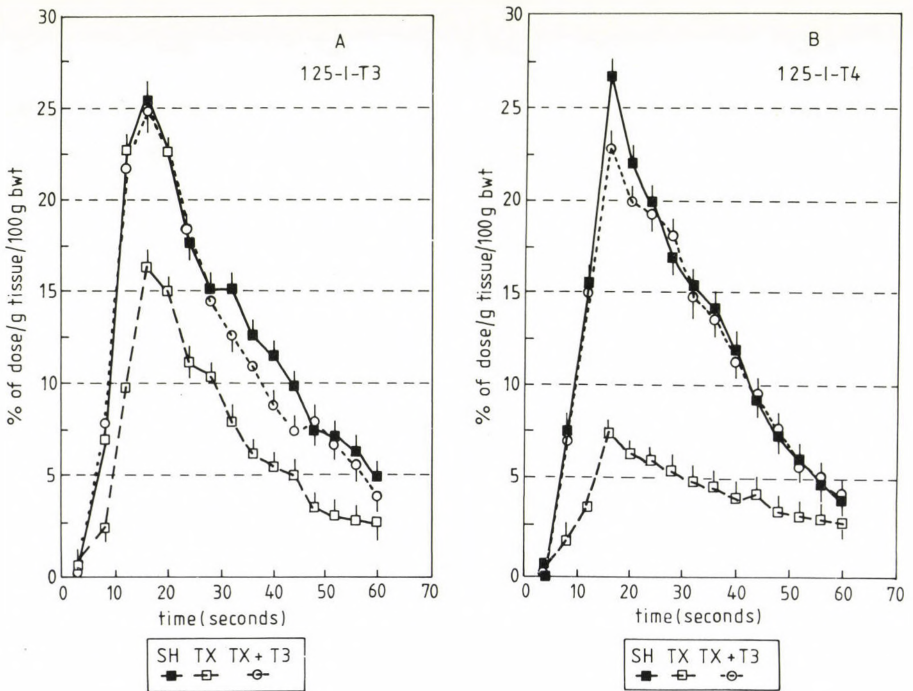

Fig. 2. The concentration of labelled T3 (panel A) and labelled T4 (panel B) in the blood plasma of the jugular vein of sham-operated control (SH), surgically thyroidectomized (TX) and thyroidectomized plus triiodothyronine supplemented (TX+T3) chickens. Samples were taken at 4-sec intervals for 60 sec. Mean ± SEM (n=5)

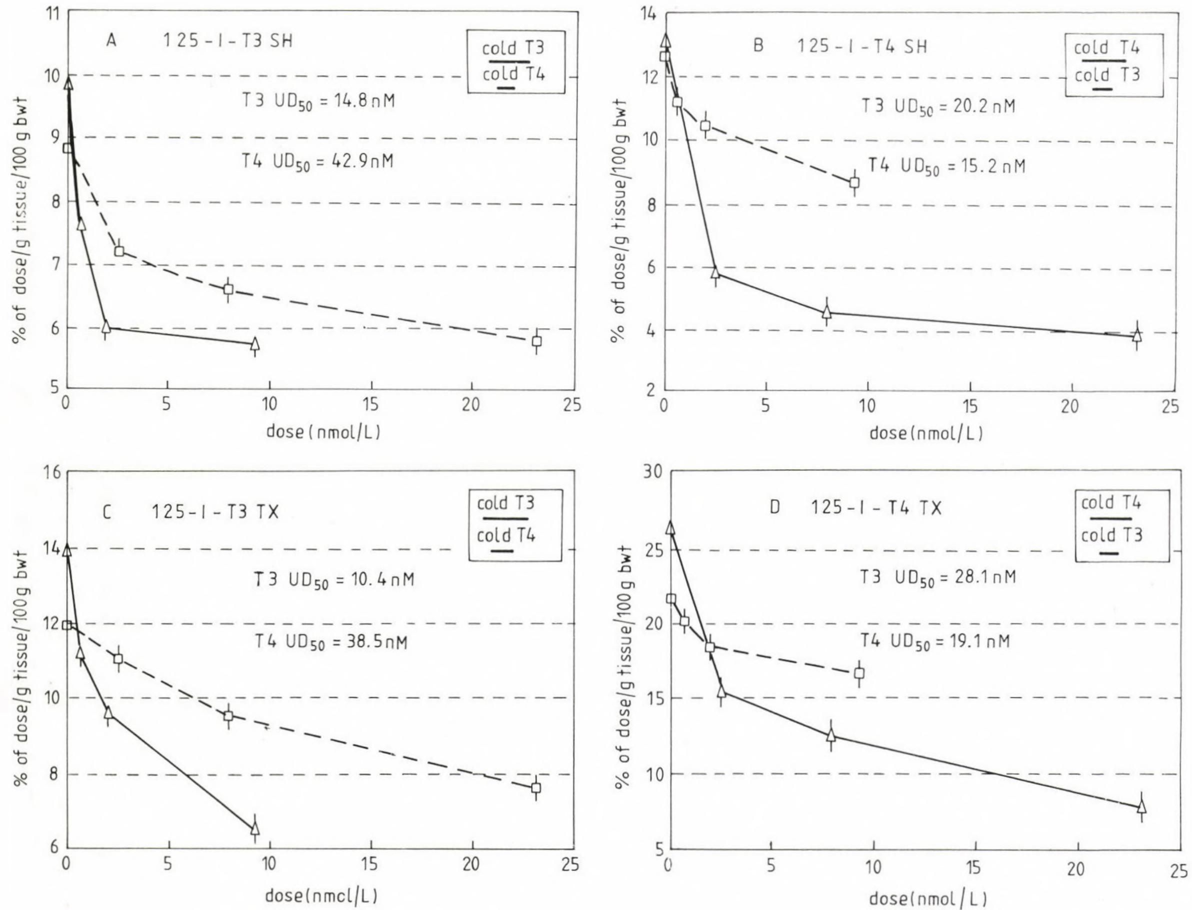

Fig. 3. The concentration of labelled T3 and labelled T4 in the hyperstriatum of sham-operated control (SH) and surgically thyroidectomized (TX+T3) chickens in the presence of increasing amounts of unlabelled T3 or T4 in the injected bolus. UD_{50} is the concentration of the cold hormone that would decrease the tissue concentration by 50 per cent. Panel A: 125-I-T3 controls (SH); panel B: 125-I-T4 controls (SH); panel C: 125-I-T3 thyroidectomized (TX); panel D: 1325-I-T4 thyroidectomized (SH). Mean ± SEM (n=5)

In Experiment 1 the uptake of iodothyronines was determined in the absence of binding proteins and cold thyroid hormones. These uptake values are 20 to 30 per cent higher than those obtained in the presence of natural binding proteins (Experiment 3). Similar results were obtained by us in earlier experiments with different proteins and protein concentrations (Rudas, 1991) and by others (Terasaki and Pardridge, 1987; Whitten and Ferguspon, 1990) as well. This points to the formerly emphasized role of the binding proteins in the regulation of hormone uptake (Mendel and Cavalieri, 1988). The enhanced uptake in the absence of binding proteins speaks in favour of the free hormone concept (Ekins, 1986). If only the free fraction had been taken up in the very short passage time applied in our experiment, one would have expected a greater than 30 per cent decrease of the uptake in the presence of binding proteins. Therefore it is reasonable to suppose that a considerable amount of the protein-bound hormone is taken up. This does not interfere with the free hormone concept (Robbins and Rall, 1960), but strengthens the possibility of a rapid replenishment of the free pool, which was several times substantiated by Pardridge (1981) and also shown by Robbins and Johnson (1982). The thyroid hormone transit time in the brain is about 1 sec (Larsen et al., 1981) and the half-life of dissociation of T3 from the surface of albumin (the major binding protein in the chicken) is close to this value, e.g. 1.1 sec for bovine serum albumin (Whitten and Ferguson, 1990). Thus about 50 per cent of the albumin-bound fraction may be transferred into the absorbable free compartment in about one sec. In addition to this, it is not yet known to what extent protein-bound hormone–capillary wall interaction may contribute to the transfer of hormone into the brain as it was shown for the liver and the kidney (Keller et al., 1969; Whitten and Ferguson, 1990). The clear difference existing in the uptake of iodothyronines between sham-operated and thyroidectomized groups of chickens speaks in favour of our former supposition (Rudas, 1988) that in the chicken not only the deiodination system adapts to hypothyroidism but also the ways by which thyroid hormones enter the tissues. The thyroid hormone supplementation technique used by our group here and before (Kühn et al., 1984) further verifies the specificity of the effect of thyroidectomy.

The determination of *in vitro* binding characteristics of thyroid hormones to plasma membranes from different tissues revealed two basic types of binding sites: one with high affinity for T4 and T3 (apparent dis-

sociation constant, Kd: 10–200 nM), and one with low affinity (Kd: 1–15 μM; Gharbi-Chihi and Torressani, 1981; Segal and Ingbar, 1982; Arnott and Eastman, 1983; Little, 1984). The UD_{50} values in this study were in the nanomol/l range and therefore speak in favour of the presence of a high affinity binding site for iodothyronines in the blood–brain barrier of chickens. The lowest uptake approximated the range between 3–6 per cent of the dose. This indicates that the uptake did not stop even at high concentrations of unlabelled hormones. Although no further details of this phenomenon were looked at here, one may suppose that at least this amount of hormone is not transferred by a specific mechanism but due to free diffusion.

When the uptake of the labelled T3 was investigated in the presence of increasing amounts of cold T4 or that of labelled T4 in the presence of cold T3 the inhibition of the uptake of the label was 1.6–3.9 times smaller than when the corresponding cold hormone (i.e. cold T4 and labelled T4) was used. This suggests that in the brain of the chicken separate routes may exist for the uptake of T3 and T4.

Hypothyroidism elicits a series of adaptive mechanisms in the brain of rats and chickens (Silva and Matthews, 1984*a*,*b*; Rudas and Pethes, 1989). Deiodination, tissue to plasma hormone clearance may change in order to preserve T3 for the hypothyroid brain cells. In the present experiments an adaptation of the brain tissue to thyroidectomy influenced not only the maximal uptake of the labelled hormone but also the UD_{50} values. Further experiments are needed to clarify whether these differences may mean the appearance of a separate class of binding sites as a result of the adaptation to hypothyroidism.

We have shown that the tissue concentration of the active thyroid hormone, triiodothyronine, in the brain of young chickens remains close to normal even 7 days after thyroidectomy in spite of the almost undetectable serum T3 concentration, and that it has relatively low tissue concentrations in other organs at the same time (Rudas and Pethes, 1989). It was concluded that at least three separate mechanisms could exist that may be responsible for the above phenomenon. Firstly the changes in membrane-dependent uptake, secondly those in the elimination of the active thyroid hormone (T3) and/or of thyroxine (T4). The third possibility is the change in the intracellular handling of thyroid hormones, namely in the activity of the deiodinase enzymes. Indeed it could be shown, that the elimination of

triiodothyronine from the brain and the deiodination system in the brain of chickens are basically influenced by hypothyroidism in the chicken (Rudas, 1989). Besides elimination and deiodination a third possibility may explain such adaptation with the appropriate alteration of the uptake system that governs the availability of thyroid hormones. The present results speak in favour of the role of the uptake in the adaptation to hypothyroidism.

In summary, the present studies provide *in vivo* evidence for two facts. First, that the uptake of iodothyronines into the brain of chickens is significantly enhanced under hypothyroid concentrations. Second, that the uptake of triiodothyronine and of thyroxine are most probably regulated by specific and separate binding sites somewhere at the blood–brain barrier in chickens.

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BIOCHEMICAL EXAMINATION OF MUSCLE SAMPLES FROM PHEASANT EMBRYOS AFFECTED BY WOFATOX 50 EC (50% PARATHION-METHYL)

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On day 12 of incubation 0.4% and 4.0% aqueous emulsions of Wofatox 50 EC (50% parathion-methyl) were injected into the air space of pheasant eggs. The eggs were opened on day 23 of the incubation period and samples were obtained from both the cervical and the femoral muscles. Atrophy was detected only in the cervical muscles by light microscopic evaluation. Calcium ion concentration was higher in the cervical musculature, but this parameter did not show significant dose-dependent changes. The activity of creatine kinase (CK) was significantly decreased only in the cervical musculature. The obtained biochemical data might result from a secondary atrophy of the cervical muscles.

Key words: Teratology, pesticide, pheasant, embryo, muscle, biochemistry, parathion-methyl

In recent years marked progress has been made in the development of methods for evaluating the teratogenic potential of pesticides. The ecotoxicological examinations of pesticides have become more important. The principal aim of our investigation was to obtain new information on the toxicological aspects of the commonly used formulation, Wofatox 50 EC.

Organophosphate insecticides cause teratological effects in pheasant embryos (cyllosis, lordoscoliosis) at the concentration (0.4%) applied in plant protection practice (Déli and Várnagy, 1985; Várnagy et al., 1982; Várnagy et al., 1984).

These microscopical cervical malformations were attributable to hypoplasia or atrophy (Várnagy et al., 1982). This study was aimed at investigating the relationships between the morphological changes mentioned above and some biochemical parameters of the musculature.

Materials and methods

Wofatox 50 EC [active ingredient: 50% 0,0-dimethyl-0(4-nitrophenyl)phosphorothioate, parathion-methyl] was used (VEB Chemiekombinat Bitterfeld, GDR). Fertile eggs were collected from own breeding: *Phasianus colchicus mongolicus* et *torquatus*. The test material Parathion 20 WP [active ingredient: 20% 0,0-dimethyl-0(4-nitrophenyl)phosphorothioate, parathion] was applied as positive control agent (Bayer, FRG).

The pesticide formulations were injected (0.1 ml/egg) as aqueous emulsion or suspension into the air space of the egg on day 12 of incubation. The control fetuses were treated by the same method with distilled water.

The lower level of Wofatox 50 EC corresponded to the concentration used in plant protection practice (0.4%). The eggs were opened on day 23 of incubation. The concentration of the positive control agent was 0.3%. Malformations were counted and typed. For histological staining the haematoxylin-eosin method was applied.

In addition, to determine the probable causes of morphological alterations observed in the fetus, samples were obtained from the cervical and femoral muscles and prepared by Kovách's method (Kovách, 1958). The following parameters were determined in samples of skeletal muscles in three parallel measurements: creatine kinase (Szász et al., 1970), creatinine (Glick, 1959), Ca^{2+} (Gitelman, 1967) and Mg^{2+} (Bohuon, 1962). The experimental data obtained for the treated and control groups were analyzed by the Chi^2 test and student's *t* test (Finney, 1972).

Results

No significant differences in mean embryonic body weight were obtained between the treated and control groups. Embryo lethality was significantly higher in the Parathion 20 WP and Wofatox 50 EC (0.4 and 4.0%) groups compared with the controls, lordoscoliosis and cyllosis being the characteristic deformities.

The light microscopic results showed an expressed atrophy only in the cervical muscles at a dose level 0.4% and 4.0% of Wofatox 50 EC and 0.3% of Parathion 20 WP.

The biochemical data are summarized in Tables 1 and 2. In the cervical muscles the activity of CK was significantly decreased in all the treated groups, while in the femoral muscles it was lower than the control value only at the higher concentration of Wofatox 50 EC and in the positive control group. The quantity of creatinine in the cervical muscles was significantly higher at the 4.0% dose level of Wofatox 50 EC, whilst in the femoral musculature it did not differ significantly from the control.

Table 1

Results of biochemical tests of the femoral muscles in pheasant fetuses

Test material	No. of fetuses	Ca ion, μg/g	Mg ion, μg/g	CK^+, U/mg	Creatinine, μg/g
Control	18	66.8	85.6	0.35	12.13
P	17	98.8	73.2	0.26	11.80
W 1	18	106.4	118.4	0.41	14.09
W 2	13	138.4^a	115.6	0.25	9.66

P = Parathion 20 WP 0.3%; W 1 = Wofatox 50 EC 0.4%; W 2 = Wofatox 50 EC 4.0%;
^{a} = $P<0.05$; $^+$ = Specific activity

Table 2

Results of biochemical tests of the cervical muscles in pheasant fetuses

Test material	No. of fetuses	Ca ion, μg/g	Mg ion, μg/g	CK^+, U/mg	Creatinine, μg/g
Control	18	191.6	124.4	0.95	19.20
P	17	108.8	99.2	0.24^b	20.50
W 1	18	140.0	148.8	0.42^b	19.00
W 2	13	166.8	90.8	0.13^b	35.10^a

P = Parathion 20 WP 0.3%; W 1 = Wofatox 50 EC 0.4%; W 2 = Wofatox 50 EC 4.0%;
^{a} = $P<0.05$; ^{b} = $P<0.01$; $^+$ = Specific activity

The average levels of Ca^{2+} increased in the femoral muscles. At the 4.0% concentration of Wofatox 50 EC they were significantly higher than the control value.

The Mg^{2+} levels did not show significant differences from the control in the muscles of the treated fetuses.

Discussion

The role of Ca^{2+} in muscular function is well known, with special respect to the activation of ATP-ase. Mg ions promote the function of CK.

The lack of Mg^{2+} and/or Ca^{2+} and the predominance of Mg^{2+} inhibit the release of acetylcholine. Furthermore, it is known that neuromuscular stimulation is decreased by a relative increase of Ca^{2+} and/or Mg^{2+} concentration (Doherty, 1979; Bálint, 1978; Karsai, 1992).

Diminished creatine kinase enzyme activity caused an increase in creatinine content, which was accompanied by a decrease in creatine-P content.

Ca^{2+} concentration is higher in the cervical musculature than in the femoral muscles, a fact expressing the increased function of m. complexus major and m. longus colli. Intracellular Ca^{2+} level increased in the femoral muscle in a dose-dependent manner: this was probably an effect brought about by the pesticide stimulus (Doherty, 1979; Morava, 1982).

The inhibition of acethylcholine esterase is known to result in a permanent influx and plasmatic accumulation of Ca^{2+}, which is more expressed in the femoral musculature. The decrease of creatine kinase activity was characteristic of dose groups treated with Wofatox 50 EC.

No significant differences were found in Mg ion concentration between groups treated with different dose levels.

Creatine kinase enzyme activity diminished significantly in the cervical musculature at both doses, while with Wofatox 50 EC this tendency could be observed only in the highest dose group. The change in CK activity resulted in a diminished creatine-P content, which did not supply sufficient energy for muscular activity.

The results of these pathological processes might account for the atrophy detected by light microscopic examination in the cervical musculature.

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STUDIES ON THE TOXIC AND IMMUNOSUPPRESSIVE EFFECTS OF CADMIUM ON THE COMMON CARP*

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In the last couple of decades, research on cadmium extended from its toxicologic aspects to its role in environmental pollution. The toxicity of cadmium to different species of fish varies widely. The cadmium sensitivity of batches of common carp, the most important cultured fish species of Hungary, was studied. Lethality, histopathologic changes and effect of cadmium on the antibody response, lysozyme level and microbicidal capacity were monitored. Wide variation was found in individual sensitivity to the toxicity of cadmium applied at concentrations of 5 to 35 mg/l with 96 h exposure time and LC_{50} of 21.07 mg/l. Histopathologic changes were observed mainly in the gills, kidney and liver. Oedema of the secondary lamellae of the gills was accompanied by a decrease in the number of mucus cells that could be seen also in the skin and gut sections at high and lower concentrations of cadmium. Distension of the Bowman's capsules in the kidney and degeneration of the renal tubular and liver cells were apparent at higher concentrations of cadmium. No histopathologic change specific of cadmium was found. The mean agglutinin titre to rabbit red blood cells of carp immunized after 96-h exposure to 5 mg/l cadmium was significantly lower than in the control group. Secondary anti-*Aeromonas hydrophila*-agglutinin response of carp treated with cadmium in the same way before immunization was practically absent. Lysozyme level in the kidney decreased to less than half of the control. Microbial killing of *Microtorula albicans* (syn.: *Candida albicans*) yeast cells increased slightly. The latter two parameters did not change significantly. The diverse sensitivity of carp to cadmium toxicity is emphasized. Changes in the immunological parameters are believed to reflect a strong stress effect of cadmium on the fish, since stress has been shown to depress specific immune response and increase neutrophil activity. These observations should call more attention to the dual nature of the toxic effect exerted by cadmium: direct toxicity resulting in fish mortality and immunosuppressive effect that probably promotes disease outbreaks.

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Key words: Cadmium, common carp, toxic and immunosuppressive effect

Toxic discharges into natural waters will normally be stopped shortly if they cause fish kills. However, sublethal toxic pollution may go unnoticed for variable durations of time without alarming signs. This type of pollution may impair the health of animals in the water environment and cause losses indirectly. So it is important to know how and what kind of damage may be caused by various sublethal exposures. Common carp (*Cyprinus carpio* L.) was selected as a model since it is the most important fish in Hungary and in large areas of the world.

Sometimes it is hard to establish a lethal concentration threshold value even for a single fish species (Abel and Papoutsoglu, 1986). A publication by Rehwoldt et al. (1972) says 96-h LC_{50} for carp should be 0.24 mg/l. However, this contrasts with the magnitude of the 10–20 mg/l range defined by Abel and Papoutsoglu (1986). Therefore, it was considered essential to try to find out about toxic cadmium levels for the present study before testing the effect of any sublethal dose.

No data were found in the literature on the effect of cadmium on the immune function of carp. To see whether acute exposure of the common carp to sublethal concentration of dissolved cadmium has any detectable effect on the defence mechanism of the carp, lysozyme level, phagocytic activity and antibody response were selected as parameters.

Materials and methods

Cadmium exposure. Yearling common carp (*Cyprinus carpio*) from three farms were used. Groups of 35–50 specimens were exposed to cadmium concentrations of 5 to 35 mg/l in three runs of exposure. Cadmium concentration was adjusted to the desired levels every day. The pH of the water was between 7.4 and 7.6. Water temperature was between 17 and 19 °C. Hardness of the water was equivalent to 138 mg $CaCO_3$. Losses from each group were recorded in the same hour every day. The LC_{50} value was calculated by the integrated normal (probit) model.

Histopathology. Samples for histopathologic analysis were fixed from the gills, skin, muscle, midgut, liver and kidney in formalin. Paraffin-

embedded histologic sections were stained with haematoxylin-eosin stains. Two carp were tested from each group of the first run of exposure.

Immune functions. Effect of sublethal cadmium exposure on the immune function of carp was tested after 96-h exposure to a cadmium concentration of 5 mg/l. The fish originated mainly from the first run of exposure.

Assessment of lysozyme activity. Lysozyme activity in kidney lysates from the carp was estimated in gel diffusion test with *Micrococcus lysodeicticus* spheroplasts (Sövényi, in press).

Microbial killing. Phagocytic activity was checked *in vivo*. A standardized suspension of fresh *Microtorula albicans* (syn.: *Candida albicans*) containing 1.4 x 10^7 CFU/ml in peptone broth was injected into the anterior sac of the swimbladder. The injection volume was 1/1,000 of the body mass. The number of survivor fungi was defined by plate counts from the pooled homogenates of swimbladder, spleen and kidney. To this end, the organs were taken out after euthanasia and chopped up and homogenized in 5% Tween-80 added at 1/10 of the body mass. Parallel plate counts were done on Sabouraud plates.

Assessment of antibody production. The antibody response of carp was checked with rabbit red blood cells and *Aeromonas hydrophila* antigens that were given intraperitoneally. After cadmium exposure, 10^7 centrifuge-washed rabbit red blood cells were given and sera were collected on day 31 after immunization. A 0.5% v/v suspension of homologous rabbit red blood cells in physiological saline was used for direct agglutination antibody titration. To check antibody production in the secondary phase of the immune response also, 6 x 10^7 viable cells of *A. hydrophila* in suspension were given twice (to fish originating from the second run of exposure), on day 0 and day 38, each time after cadmium exposure. Sera were collected on day 65. Serum antibody titres were obtained with a 0.5% v/v saline suspension of cell pellet of heat-killed *A. hydrophila.*

Results

Toxicity and lethality of cadmium. Cadmium exposure generally caused excitement, hyperexcitability and abnormal tense with sudden

swimming motions. General oedema developed in the moribund fish. Attempts to establish the LC_{50} value for carp resulted in mortalities between 0 and 94.2%, with very diverse data in each run of the experiment (Table 1). For probit analysis, two extreme lethality percentages had to be omitted. The LC_{50} value was calculated from the data of a total of 630 specimens. LC_{50} was 21.07 mg/l (fiducial limits: 18.58 mg/l and 23.88 mg/l).

Histopathology. The sections from cadmium-exposed carp stained very faintly. Histopathologic changes were observed mainly in the gills, kidney and liver (Figs 1–12).

Lysozyme level. Mean lysozyme level in the kidney of carp that were exposed to 5 mg/l Cd for 96 h (797 units, S.E. 171.5) was less than half of the control (1881.2 units, S.E. 511.8). The difference was statistically non-significant.

Microbial killing. The mean number of viable *M. albicans* in the cadmium-treated fish (470.6 CFU/ml, S.E. 91.1) differed non-significantly from the control (705.4 CFU/ml, S.E. 112.7). This means that microbial killing may have increased slightly in the cadmium-exposed fish, since these numbers represent an average theoretical microbial killing of 65.6% for the cadmium-exposed carp and 48.5% for the control.

Antibody response. The results of immunization are presented in Table 2.

Discussion

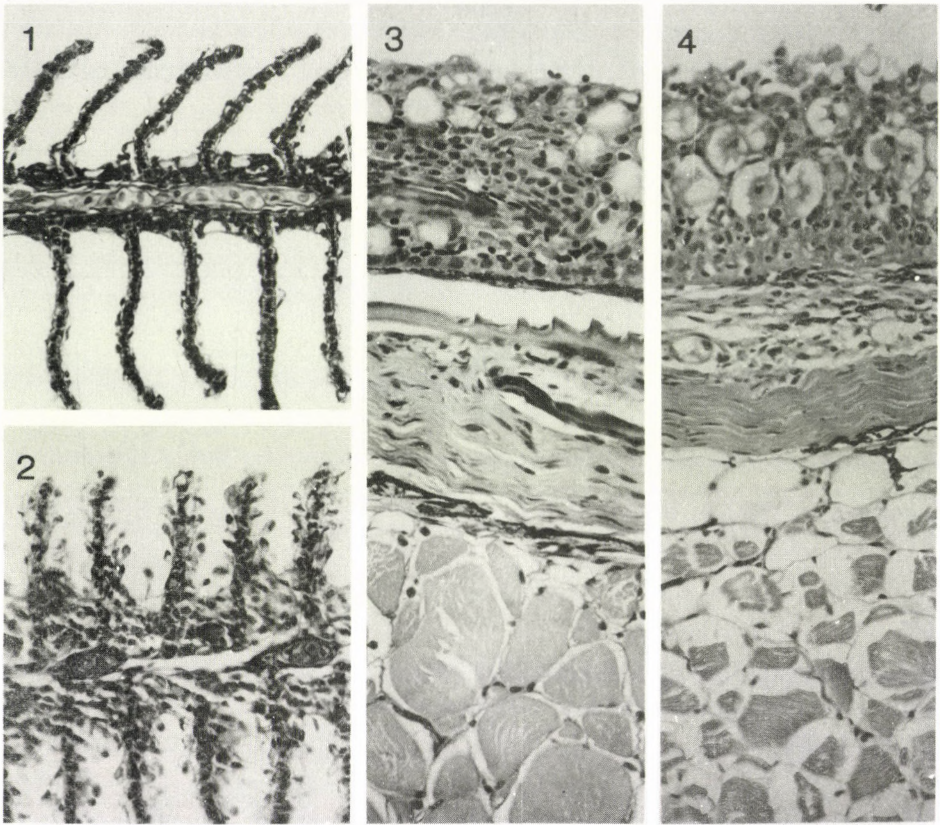
In the present study, the toxicity of cadmium showed a high diversity of mortality depending on the origin of the carp: high sensitivity was observed in the second run of toxicity testing and very low sensitivity was seen in the third one. It seems that it is more than the problem of median survival time changing very slowly over a wide range of cadmium concentrations as mentioned by Alabaster and Lloyd (1980). This can be said on the basis that the batches were kept in water of identical parameters. On the variance between batches, great individual variance of sensitivity to the toxic effect of cadmium seems to be superimposed. This complexity of variance requires more detailed studies to full understanding. Therefore, carefulness is required when applying LC_{50} values from one batch of fish to others, especially to batches of other species as it was pointed out by Abel and Papoutsoglu (1986).

Table 1

Tests to determine the 96-h LC_{50} of cadmium. Data of three runs with three respective batches of carp

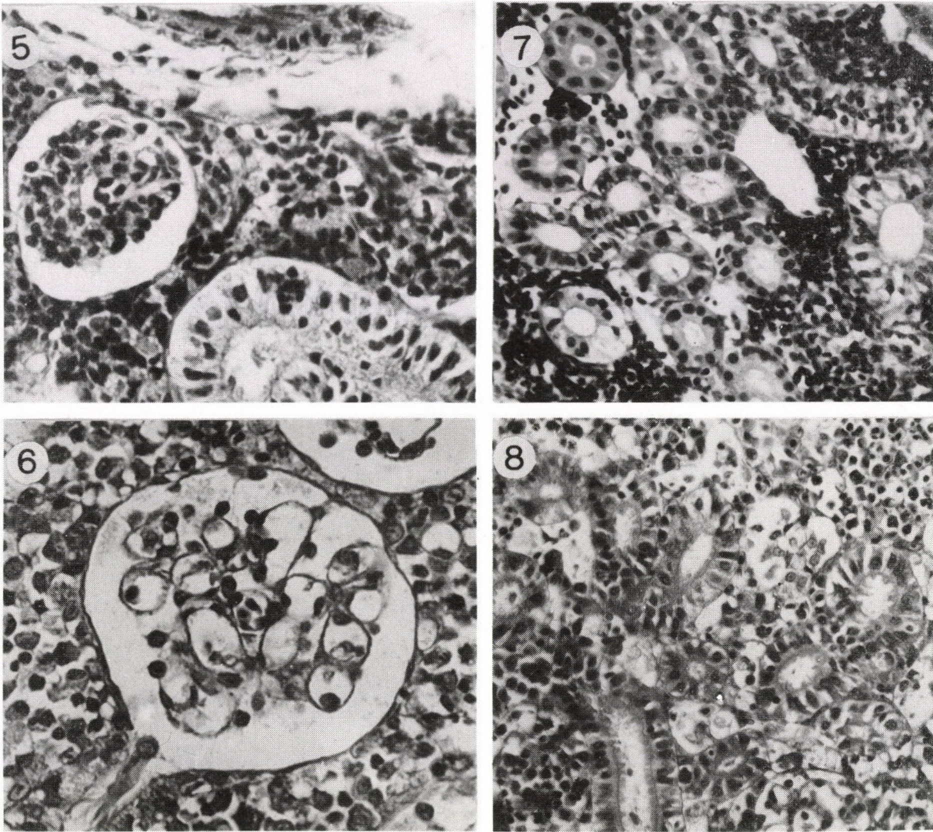
Cd concentration mg/l	n	% mortality				
		24 h	48 h	72 h	96 h	total
First run						
0	35	0	0	0	0	0
5	35	0	0	0	0	0
10	35	0	0	0	5.7	5.7
13.92	35	17.1	11.4	5.7	5.7	39.9
15	35	5.7	0	2.9	5.7	14.3
35	35	71.4	22.8	0	0	94.3
Second run						
0	56	1.8	0	0	0	1.8
5	48	0	2.1	4.2	8.3	14.6
10	52	11.5	5.8	0	1.9	19.2
15	53	9.4	11.3	7.5	3.8	32.1
20	54	1.9	9.3	1.9	1.9	14.8
25	46	13	28.3	2.2	4.3	63
Third run						
0	50	0	0	0	0	0
10	50	0	0	0	0	0
15	50	0	0	0	0	0
20	50	0	0	0	0	0
25	50	0	0	0	0	16
Summarized data of mortality percentages						
0	141	0.6	0	0	0	0.6+
5	83	0	1.0	2.1	4.1	7.3+
10	137	3.8	1.9	0	6.3	12.1+
13.92	35	17.1	11.4	5.7	5.7	39.9
15	138	8.8	3.7	5.4	6.9	24.9+
20	104	0.9	4.6	0.9	0.9	7.5
25	96	6.5	14.1	6.2	8.3	35.1+
35	35	71.4	22.8	0	0	94.2+

+: data used in the probit analysis



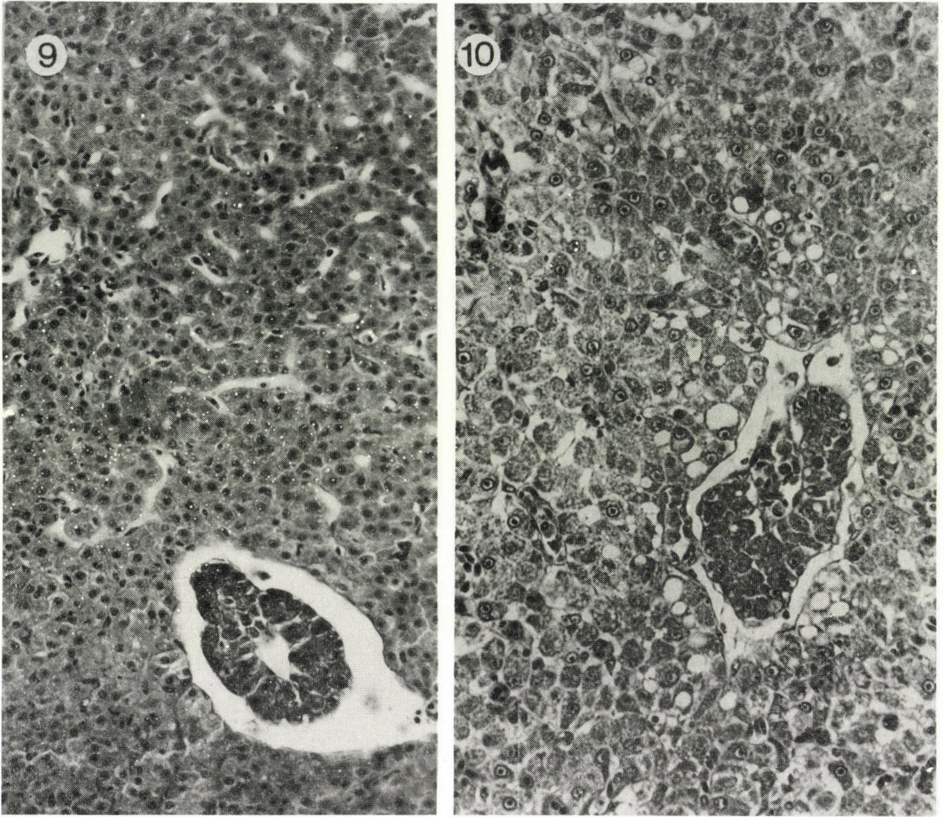
Figs 1 and 2. Gill sections from a control and a moribund carp after cadmium exposure, respectively. Pathology: oedematous distension, detachment of epithelial cells or cell layer from the secondary gill filaments, hyperplasia of the interlamellar epithelium. Haematoxylin and eosin (HE), ×370

Figs 3 and 4. Sections of skin and dorsal muscles from a control and a moribund carp after cadmium exposure, respectively. Pathology: great increase in the number of club cells, loose connective tissue and degeneration of muscle cells

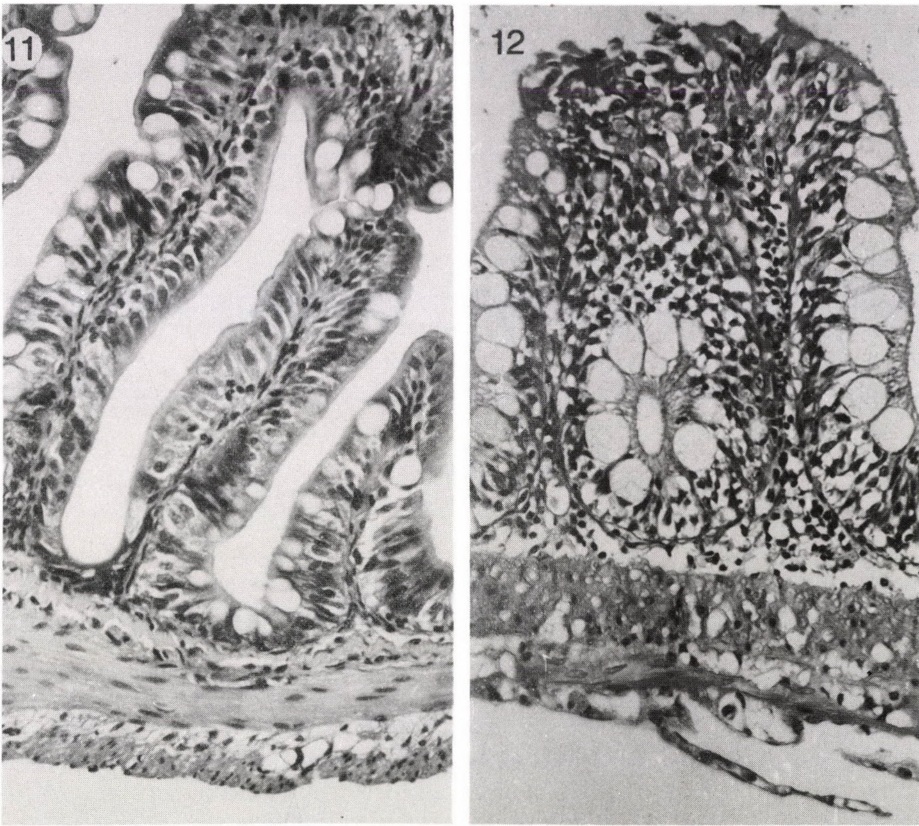


Figs 5 and 6. Kidney sections from a control and a moribund carp after cadmium exposure, respectively. Pathology: ectasy of Bowman's capsules and their capillaries. HE, ×740

Figs 7 and 8. Hydropic degeneration of tubular epithelia, oedema. HE, ×370



Figs 9 and 10. Liver sections from a control and a moribund carp after cadmium exposure, respectively. Pathology: hyperaemia, oedema, grave enlargement of the Disse-spaces, degeneration of parenchymal cells and pancreatic cells. HE, ×370



Figs 11 and 12. Sections of midgut from a control and a moribund carp after cadmium exposure, respectively. Pathology: oedema in the gut wall, swelling of the epithelium, distasia of epithelial cells, abnormal brush borders, few and enormous mucus cells. HE, ×370

Table 2

Agglutinating antibody titres found on the day of immunization and after immunization. The differences of the means of a respect are significant

Group	n	Mean titre	S.E.	Mean titre	S.E.
Primary response to rabbit red blood cells					
		day 0		day 31	
Cd treated	15	11.5	2.6	315.7	62.3
Control	16	5.9	1.3	672.0	116.6
Secondary response to *Aeromonas hydrophila*					
		day 0		day 65	
Cd treated	36	2.1	2.2	5.4	3.5
Control	48	2.1	2.2	48.6	8.1

Cadmium exposure resulted in a similar LC_{50} value (21.07 mg/l) for carp as that reported by Abel and Papoutsoglu (1986), which is much higher than the 0.24 mg/l LC_{50} value published by Rehwoldt et al. (1972). Attempts to explain the differences fail on the fact that the toxicity of cadmium to fish is influenced by factors like temperature (Eisler, 1971), level of dissolved oxygen (Voyer, 1975), water hardness (Calamari et al., 1980) and pH (Cusimano and Brakke, 1986) that are not all characterized or comparable in our cases.

The histopathological signs described here largely coincide with those described elsewhere for other fish species by Gardner and Yevich (1970) and Cearley and Coleman (1974). These changes are interpreted to be concentrated around the membrane damages of various tissue cells, which explains oedema, an osmoregulatory disorder. Failure of other metabolic functions bound to membrane structures should also be considered among the consequences.Previously, it was claimed by Schreck and Lorz (1978) that cadmium is toxic but it does not affect trout as a stressor at 12 µg/l concentration. However, since the cadmium concentration was much higher in this study, it is thought that the tissue damages listed above imposed a heavy metabolic stress on the carp. It is considered that the

changes observed in immunological parameters can be also explained easily as a tendency of the immune reactions shifting to stress type responses.

Though lysozyme level did not change significantly in the cadmium-treated carp as compared to the control, the decrease indicates a strong negative tendency of lysozyme activity in the kidney. Lysozyme is a product mainly of neutrophil and macrophage cells and its level increases together with the numerical changes of these cells. Possibly, the lysozyme level tended to decrease either because of a functional damage to the protein metabolism of these cells or as a result of the decrease of neutrophil cell counts in the kidney haemopoietic tissue because of the stress as reported by Peters and Schwarzer (1985). More data are needed to support any of these assumptions.

Stress reduces recruitment of leucocytes at sites of injury. This is normally the result of decreased interleukin release by other leucocytes. It does not, however, reduce ability of microbial killing of phagocytes. Since peptone, which is a chemotactic attractant for migrating leucocytes, was used to disperse *M. albicans* injected to the carp recruitment decrease after stress is supposed to be overcome. It seems reasonable to say that the increase of microbial killing in the cadmium-treated fish is related to the greater number of circulating neutrophils and macrophage-like cells, which was shown to occur with stress by Peters and Schwarzer (1985) and Ellaesser and Clem (1986). In this study, significant difference between the cadmium-treated and control fish was detected in the extent of their antibody production. This result corroborates those of O'Neill (1981). He found that antibody production to MS2 phage in brown trout was significantly lower after a single dose of intraperitoneal cadmium. As an explanation, he referred to interactions of cadmium with a range of biologically active molecules, antibody among them. However, it is considered that immunosuppression may be a result of a stress effect, also since it has been shown in fish by Muiswinkel and Ginkel (1981) that stress depresses specific immune response.

An examination of cortisol level should decide whether or not stress effect develops in carp after exposure to sublethal cadmium levels. If it does, stress should be reckoned with as a factor further increasing the chance of disease outbreaks (Wedemeyer, 1974). In any case, it seems necessary to grow awareness about the dual nature of cadmium damage to the living environment. One is the obvious direct lethal effect and the other is a

sublethal effect: immunosuppression and, perhaps, stress. Mortalities initiated by the sublethal effect may not be less damaging or dangerous to the fish stocks than direct toxicity except that their primary aetiology will not be as obvious.

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T-CELL DEFICIENCY IN SUFFOLK LAMBS

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In a flock of Suffolk sheep respiratory diseases were regularly observed, while a flock of Booroola sheep kept under similar feeding and management conditions remained healthy. Experiments were conducted to compare the immunological and haematological parameters of Suffolk and Booroola sheep of different age groups. The percentage of T and B cells in the lymphocyte population and the capacity for blastogenesis induced by nonspecific mitogens were analyzed. Suffolk sucking lambs had significantly ($P<0.001$) lower Concanavalin A (Con-A) induced blastogenesis and significantly lower T cell percentage at 6 months of age than Booroola lambs of the same age. B cell percentage and the rate of blastogenesis induced by *Phaseolus vulgaris* lectin (PHA) were lower in Suffolk lambs, though the differences were not significant. Sucking and growing Suffolk lambs had significantly ($P<0.01$) lower red blood cell count and packed cell volume than Booroola lambs of the same age. Blood haemoglobin concentration was also lower in Suffolk lambs. No consistent differences were seen between the two breeds in total leucocyte count. T-cell deficiency and anaemia could be corrected by treatment with the immunomodulator levamisole (administered at a dose of 3 mg/kg body mass intramuscularly, twice with an interval of 10 days) and with vitamin B_{12} (1,000 μg/animal i.m.), respectively. On day 16 after the first treatment, the T-cell percentage, Con-A induced blastogenesis, red blood cell count, and packed cell volume of growing Suffolk lambs increased. T-cell deficiency and anaemia, either separately or together, may explain the lower resistance of Suffolk lambs to opportunistic pathogens. The relative contribution of genetic and environmental factors requires further studies.

Key words: Immunodeficiency, anaemia, Suffolk lambs

According to observations made in recent years, a substantial proportion of losses occurring in newborn or young animals can be attributed to the defective or impaired functioning of the immune system. Animals with an insufficiently functioning immune system have weaker resistance, and are easily affected even by pathogens that cannot produce disease in

individuals of normal immune status (Banks, 1982). Of the hereditary immunodeficiencies, the B-cell form has been reported from domestic animal species (Perryman, 1982). Concerning the immunodeficiency attributable to impaired functioning of the T cells only hypothetical data are available (Tizard, 1984).

In human medicine, efforts to normalize the immune mechanisms harmful to the organism are steadily gaining ground (Gergely, 1980). One of the drugs suitable for this purpose is levamisole [L-(-)-2,3,5,6-tetrahydro-6-6-phenylimidazole-(2,1-b)-thiazole hydrochloride], which up to 1971 had been known as an anthelmintic compound. When applied at fractions of its usual dose, levamisole exerts immunomodulating effect (Renoux and Renoux, 1971; Symoens et al., 1979). In immunodeficiencies of various origin, levamisole enhances the T-cell-mediated immune response and stimulates the phagocytic activity and maturation of macrophages and polymorphonuclear leucocytes (Pár, 1977; Symoens and Rosenthal, 1977; Symoens et al., 1979; Brunner and Muscoplat, 1980). Levamisole has been reported to exert a favourable immunomodulating effect in domestic animals when used together with attenuated bovine herpesvirus-1 vaccine in calves (Babiuk and Misra, 1982) and polyvalent clostridium vaccine in sheep (Hogarth-Scott et al., 1980; Bineva et al., 1987). In sucking piglets, Misley and Sárközy (1979) demonstrated the influence of levamisole on the cell-mediated immune response by the 2,4-dinitro-chlorobenzene skin test.

The ontogeny of the immune system and the development of immunocompetence in sheep have been studied by several authors (Silverstein and Prendergast, 1970; Jordan, 1976; Trnka and Moris, 1985; Tuboly et al., 1984). To the best of our knowledge, however, B- and T-cell immunodeficiency of sheep has not been reported yet.

In this work, the Suffolk and the Booroola sheep breeds were compared in respect of immunological and haematological status. The study was prompted by the observation that Suffolk sheep kept in the same place, under identical management and feeding conditions as Booroola sheep, developed respiratory disease which became permanent.

Materials and methods

Animals

The Suffolk breeding sheep were imported from an area of continental climate as adult animals. The Booroola sheep were derived from embryo import. The health status of progeny flocks derived from these animals differed even though they were kept in the same place. In the Suffolk sheep a respiratory disease caused by adeno- and reoviruses occurred. By bacteriological examination of the nasal secretions and lung samples showing gross lesions *Pasteurella haemolytica* infection could be established. Despite the treatments, the respiratory disease became persistent and posed a risk especially to young animals in the spring and autumn months. As the Booroola sheep did not exhibit such signs, comparative immunological and haematological studies were performed to elucidate the breed disposition, if any. These studies involved a total of 40 Suffolk and 95 Booroola sheep born in the same place (6- to 10-week old animals) and kept on the same pasture (6-month-old sheep).

At the beginning of the study, nasal swab samples taken from adult sheep showing upper respiratory signs revealed a rich Gram-negative microflora and cocci without the presence of *Pasteurella haemolytica*. Serologically the flocks were free from antibodies to *Brucella abortus ovis, Leptospira pomona, L. hardjoe, L. grippotyphosa, Chlamydia* and *Salmonella abortus ovis.*

At one-day-old and then at 4 weeks of age the lambs received vitamins A, D_3, E and selenium treatment. To provide them with specific protection to adenoviruses, they were vaccinated with Ovivac (Phylaxia). During the rearing period the lambs were fed a preventive dose of the antiparasitic preparation Vermitan granules (Chinoin) mixed in the feed.

Laboratory examinations

Blood samples for immunological and haematological tests were collected from the jugular vein into two tubes, one containing 25 μl heparin and the other 0.1 ml dipotassium ethylenediamine tetraacetate (EDTA). The samples were analyzed within 3 h of collection.

Immunological tests

For the *in vitro* tests, lymphocytes were separated by Ficoll-Paque gradient centrifugation. After checking their viability, 10^6 cells per cm^3 were suspended in Hanks' solution containing 10% fetal calf serum.

The lymphocyte stimulation test (LST) was performed according to Outteridge et al. (1981). The percentage of T cells in the lymphocyte population was determined by a minor modification of the method described by Mueller et al. (1979) and Salmon et al. (1987), and that of B cells as described by Rose and Bigazzi (1973).

Haematological tests

Haematological variables were determined by a PHA-1 type automatic apparatus (Medicor, Budapest), using a Redcal-4 standard for calibration. Total red blood cel! (RBC) and white blood cell (WBC) counts, haemoglobin concentration (Hb) and packed cell volume (PCV) were determined, and mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH) and mean corpuscular haemoglobin concentration (MCHC) were calculated instrumentally.

Blood smears were prepared on slides and stained by the rotuine Pappenheim method for morphological examination. One hundred leucocytes per sample were counted for determining the percentage of lymphocytes, neutrophils and other cell types.

Drugs

The majority of 6-month-old Suffolk lambs exhibited upper respiratory signs. One group of Suffolk sheep was treated intramuscularly with 3.0 mg/bwkg levamisole (levamisole 7.5% inj., Chemical Works of Gedeon Richter Ltd., Budapest) at 10-day intervals, and with 1,000 μg vitamin B_{12} injection (Chemical Works of Gedeon Richter Ltd., Budapest) at the time of the first treatment. The other group received exclusively vitamin B_{12} injection (1,000 μg). On day 16 after the first treatment, i.e. on day 6 after the second levamisole treatment, the immunological and haematological tests were repeated in the two groups of Suffolk lambs that received different treatment.

Results

The results of immunological tests in 6- to 10-week-old and 6-month-old Suffolk and Booroola sheep are shown in Table 1. Blastogenic transformation of lymphocytes stimulated with PHA (*Phaseolus vulgaris* lectin=agglutinin) and the percentage of B lymphocytes were in the physiological range both in the Suffolk and in the Booroola lambs, but the values were lower in the Suffolk lambs at both ages. In addition, blastogenesis stimulated with the mitogen Con-A in 6- to 10-week-old Suffolk lambs was only 66% of the values measured in Booroola lambs of the same age ($P<0.001$). In growing (6-month-old) lambs the percentage of T lymphocytes was only 76% of that obtained in Booroola sheep of the same age ($P<0.001$). After the treatment of experimental groups of Suffolk sheep it was found that the LST value and T cell percentage of lambs given levamisole therapy had increased by 10% ($P<0.001$). No significant change was found for Suffolk lambs not treated with levamisole (Table 2).

Table 1

Comparison of immunological variables of Suffolk and Booroola lambs

Immunological variable*	6–10 weeks			6 months		
	Suffolk (n = 13)	Booroola (n = 10)	P	Suffolk (n = 16)	Booroola (n = 16)	P
Con-A	28.3	42.0	0.001	40.3	45.6	0.001
T	53.5	57.0	-	49.2	64.8	0.001
PHA	32.0	38.4	0.001	38.0	41.0	0.01
B	24.0	30.0	0.001	31.0	35.0	0.01

* Mitogen-induced blastogenesis and percentage of T and B lymphocytes

Comparative examination of the haematological variables (Table 3) showed a significantly ($P<0.01$–0.05) lower RBC count in the Suffolk sheep than in Booroola sheep of the same age. As compared to data reported in the literature as being typical of the species and the age group, the RBC count was lower than normal (Schalm and Jain, 1986). No difference was found between the two breeds in the WBC count which corresponded to the physiological values. In connection with the RBC count, the PCV was also lower in Suffolk sheep ($P<0.01$–0.001), showing a value

as low as 12%. At the same time, MCHC and MCH were significantly ($P<0.001$) higher, primarily in the 6-month-old animals.

The examination repeated on day 16 after vitamin B_{12} treatment revealed a significant improvement in RBC count, PCV, MCH and MCHC values (Table 4). The differential blood count showed a nearly 1:1 ratio of neutrophilic granulocytes and lymphocytes in both breeds. In none of the cases did the proportion of eosinophilic granulocytes exceed 3%, which confirms that the flocks examined were free from parasites (Table 5).

Table 2

The effectiveness of levamisole and/or vitamin B_{12} treatment in experimental Suffolk lambs

Immunological variable	6–10 weeks		6 months			
	Suffolk* (n = 5)	Suffolk** (n = 5)	Suffolk* (n = 5)	Suffolk** (n = 5)	P*	P**
Con-A	42.0	38.6	42.6	48.8	-	0.001
T	50.8	49.4	51.8	59.2	-	0.001

* treated with vitamin B_{12} ; ** treated with vitamin B_{12} plus levamisole

Table 3

Comparison of haematological variables of Suffolk and Booroola lambs

Haematological variable	6–10 weeks			6 months		
	Suffolk (n = 9)	Booroola (n = 7)	P	Suffolk (n = 19)	Booroola (n = 19)	P
RBC (10^{12}/l)	2.860	4.040	0.01	2.660	3.69	0.001
Hb (mmol/l)	7.060	7.610	-	6.830	7.15	-
PCV (l/l)	0.128	0.184	0.01	0.122	0.17	0.001
MCV (fl)	45.000	45.600	0.05	45.800	46.30	0.001
MCH (fmol)	2.380	2.010	-	2.560	1.95	0.001
MCHC (mmol/l)	53.10	41.400	0.05	55.900	42.30	0.001
WBC (10^9/l)	6.160	6.210	-	9.920	8.87	-

Table 4

Effect of treatments on haematological variables in Suffolk lambs

Haematological variable	Before treatment		After treatment			
	Suffolk* (n = 5)	Suffolk** (n = 5)	Suffolk* (n = 5)	Suffolk** (n = 5)	P*	P**
RBC (10^{12}/l)	2.690	2.290	3.200	3.390	0.050	0.001
Hb (mmol/l)	6.610	6.260	6.210	6.640	-	0.010
PCV (l/l)	0.123	0.105	0.151	0.161	0.050	0.001
MCV (fl)	45.700	46.200	47.000	47.000	0.001	-
MCH (fmol)	2.490	2.800	1.950	2.030	0.010	0.010
MCHC (mmol/l)	54.50	61.300	41.400	43.000	0.010	0.010
WBC (10^9/l)	9.040	9.300	8.320	8.360	-	-

* treated by vitamin B_{12} ; ** treated by vitamin B_{12} plus levamisole

Table 5

Effect of age on the leucocyte types of Suffolk and Booroola lambs

Age	Breed (n)	Ne %	Ly %	Mo %	Eo %	Ba %
6–10 weeks	Suffolk (6)	52.8	42.5	2.5	1.30	0.9
	Booroola (6)	52.5	45.0	1.5	0.82	0.2
6 months	Suffolk (10)	50.9	48.5	0.5	0.10	-
	Booroola (10)	48.7	49.8	1.0	0.40	0.1

Ne=Neutrophils; Ly=Lymphocytes; Mo=Monocytes; Eo=Eosinophils; Ba=Basophils

Discussion

Besides factors forcing animals to adapt, different pathogens may also play a role in the acquired forms of immunodeficiency (Perryman, 1979; McGuire et al., 1981). Facultative pathogens can cause disease only in an organism of impaired resistance. The aim of this study was, therefore, to determine if there exist differences in the immunological status and haematological variables between two sheep breeds of different breeding pur-

pose but kept under identical managemental conditions and, if yes, to see if these differences are indicative of an impaired resistance in Suffolk sheep.

In sheep, the results of *in vitro* LST showed that PHA-stimulated blastogenesis primarily induces the nonspecific blastogenic transformation of B cells, while Con-A causes that of T cells (Outteridge et al., 1981). In this experiment, the rate of PHA-stimulated blastogenesis and the number of B lymphocytes were in the physiological range in both the Suffolk and the Booroola sheep (Al Salami et al., 1985; Lascelles et al., 1985). Under physiological conditions, the T cells represent approx. 60% of the peripheral lymphocytes in lambs (Ezaki et al., 1985). The same was found for Booroola sheep, while in Suffolk lambs the index of Con-A induced blastogenesis and in Suffolk growing sheep the number of T cells was significantly (P<0.001) lower.

The results demonstrate that an immunodeficiency existed in the Suffolk lambs (Table 1). The type of immunodeficiency found in these animals manifests itself in the lower number of T cells and in reduced reactivity to Con-A. In calves, Le Jan (1981) found that levamisole administered at a dose of 7 mg/kg body mass enhanced the *in vitro* reactivity of white blood cells to nonspecific mitogens for five days after the treatment. In our experiment, an indirect proof of T-cell immunodeficiency in Suffolk sheep was that after treatment with the immunomodulant levamisole (administered at a dose of 3 mg/kg body mass) both the number of T cells and their reactivity to Con-A significantly (P<0.001) increased (Table 2). Clinically, the severity of the upper respiratory signs decreased. The haematological variables of Suffolk sheep of high growth rate were found to be less favourable than those of Booroola sheep of the same age and kept under identical management conditions.

Further studies are needed to elucidate whether the T-cell immunodeficiency found in Suffolk sheep is genetically determined or caused by other factors.

Acknowledgements

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BOOK REVIEWS

J. FEHÉR, A. BLÁZOVICS, B. MATKOVICS and M. MÉZES: *Role of Free Radicals in Biological Systems.* Akadémiai Kiadó, Budapest, 1993. 252 pages. ISBN 963 05 6501 3.

Hungarian experts involved in free radical research held their fourth congress at Balatonaliga in May 1991. This book is the Proceedings of that three-day conference which has reached international status in the meantime. It contains the material of the most important papers in a comprehensive form. Among the authors we can find researchers (biologists, botanists, agricultural engineers, physicians and veterinarians) who study the role of free radicals in diverse fields of biology. The presented research results demonstrate the importance of free radicals in physiological and pathological processes in all fields of biology. The 252-page book contains the material of 30 papers, most of which are illustrated by figures and tables. A detailed reference list can be found at the end of each paper.

New scientific information is presented on the role of free radicals in physiological processes such as the germination of plants, the function of phagocytes, or the immune response. Beyond these fields, the book offers us new information primarily on free radical reactions involved in pathological biological processes.

Several papers deal with the potential therapeutic uses of plants of antioxidant properties. These reports provide new data also for veterinarians. The Proceedings can be used equally well by theoretical and practical specialists dealing with free radical research. It is a pity that the book came out only two years after the congress, with support from the Liver Research Foundation. We can only hope that the proceedings of the fifth free radical congress that took place in Pécs in February 1993 will be published in a shorter time.

Tibor GAÁL

G. CSOMÓS and J. FEHÉR: *Free Radicals and the Liver.* Springer Verlag, Berlin – Heidelberg – New York – London – Paris – Tokyo – Hong Kong – Barcelona – Budapest, 1993. 157 pages, with 85 illustrations. ISBN 3-540-54445-3.

This 157-page, hardcover volume contains the abstracts of papers read at the latest congress of the European Association for the Study of the Liver (EASL), held in Budapest. Thirty well-known researchers from Hungary, Germany, Italy and the United States present, in ten summaries, their scientific findings obtained on the development and treatment possibilities of toxic liver disorders. Studies in recent years have shown that free radicals play an important role in toxic liver disorders. This finding is supported by the observations made by the contributors of the present volume. Two major areas discussed: the pathogenesis and pathophysiology of processes induced in the liver by free radicals, and the therapeutic possibilities. The importance of free radicals in toxic liver disorders, such as e.g. carbon tetrachloride, alloxan and paraquat poisoning well known to veterinarians, has been demonstrated by model experiments. The risk of free radical reactions that accompany the use of certain drugs (adriamycin, bleomycin, etc.) is reported along with the side effects arising from the incorrect use of the inhalation anaesthetic agent halothane. The relationship between lipid peroxidation processes and hepatocyte damage, the hepatotoxic effect of iron load, and the role of free radicals in the development of experimental fatty liver syndrome are reported.

The drugs, first of all the antioxidant preparations, used in the struggle against toxic liver disorders are also described in detail. The therapeutic results published have been derived from experiments on rats as model animals and from studies conducted on a human patient material. Despite the fact that the volume is not intended specifically for veterinarians, and that its authors are physicians and biologists, it can be recommended both for veterinary researchers studying the development of toxic liver disorders and for veterinary practitioners and students interested in recent advances in the therapy of liver diseases.

Tibor GAÁL

Manual of Standards for Diagnostic Tests and Vaccines for Lists A and B Diseases of Mammals, Birds and Bees. Second edition. Office International des Épizooties, Paris, 1992. 783 pages.

The Office International des Épizooties (O.I.E.) published, in a single volume, the English-language work published between 1989 and 1991 in three volumes under the title "Recommended Diagnostic Techniques and Requirements for Biological Products".

The first edition published by the O.I.E. met with a huge success and has become an indispensable manual for veterinary diagnosticians, vaccine production and control specialists. The second edition has added to the substantial values of the first edition: the single volume is easier to handle, the title is more apt and precise. The first edition has been expanded with a single but spacious chapter: the precise list of O.I.E. reference laboratories.

The book consists of a general and a specific part.

The general part comprises three short, comprehensive chapters. The first chapter discusses the taking of samples for different tests. It provides the reader with useful information about sampling procedures from different organs and the submission of samples. The second chapter summarises the rules of testing different biological products for sterility. The information presented in the third chapter facilitates the safe operation of veterinary microbiological laboratories. Specialists working in this field find that the problem of biological hazard is becoming increasingly important. The book facilitates the development of laboratory safety by conveying useful information.

In the specific part, the diagnostic and vaccine production methods of 15 diseases included in O.I.E. List A and 78 diseases included in List B are presented.

The book was written by a large international staff of experts. The material of each disease was compiled by the best international specialists of the given field.

A great asset of the book is the consistency of editorial work. The same editorial policy is followed throughout.

Each chapter begins with a short (usually 1–1.5-page) summary of the given topic. This is followed by *Chapter A*, which gives a brief summary of the disease and then describes the diagnostic methods. The diagnostic part always begins with identification of the pathogen, followed by the description of the serological (and other) testing methods. *Chapter B* deals with the requirements of biological products, in an arrangement corresponding to the seed lot concept, the vaccine production practice of our day.

The *Seed Management* section defines the fundamental criteria of Master Seed materials used for vaccine production. The second part of the chapter, entitled *Manufacture*, summarises the most important elements of product manufacture.

The section entitled *In process control* contains the methods of testing vaccines for sterility, safety and stability, and the system of relevant criteria.

The last section of Chapter B is *Batch control.* It describes the methods of controlling the sterility, safety and efficacy of vaccines and the requirement levels relating to the given method.

Recently the problems of registering and controlling vaccines and drugs have become increasingly important in the animal health activity of the highly developed countries.

This O.I.E. publication is an indispensable aid to developing the standard principles of registration and to harmonising the methods and requirements of registration in the different countries.

The chapter is concluded by a list of the most important publications concerning the diagnostics of the given disease and the production as well as control of biological products developed against it.

The book has been made with a practical and neat printing technology which facilitates the enforcement of consistent and logical editing principles and makes the book a reading matter of clear arrangement.

It can perhaps be stated without exaggeration that this technical book unique in this field is indispensable for the specialists of diagnostic institutions. It can be used also by veterinary practitioners working in the field of therapy and prevention.

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CONTENTS

Animal nutrition

Animal models in experimental atherosclerosis: A critical review. *Fekete, S.* 3
Nutritional factors affecting milk quality, with especial regard to milk protein: A review. *Hullár, I.* and *Brand, A.* 11
The influence of inedible raw material rendering method on meat meal quality. *Ristić, M., Kormanjoš, Š., Ćurčić, R.* and *Pupavac, Veselina* 33
Complex study of the physiological role of aluminium. I. Review of the literature. *Bokori, J.* and *Fekete, S.* 193
Complex study of the physiological role of aluminium. II. Aluminium tolerance tests in broiler chickens. *Bokori, J., Fekete, S., Kádár, I., Vetési, F.* and *Albert, M.* 235
The role of feed protein quality in reducing environmental pollution by lowering nitrogen excretion I. Survey of principles in protein evaluation for monogastrics: A review. *Hegedűs, M.* 265
The role of feed protein quality in reducing environmental pollution by lowering nitrogen excretion II. Choice of an assay for monitoring protein quality and predicting protein utilization. *Hegedűs, M.* 283

Animal reproduction

Differences in biochemical profile from active and inactive ovarian tissue in relation to oestrous cycle phases. *Sureshkumar, P. K.* and *Janakiraman, K.* 301

Bacteriology

Treatment of calf pneumonia with tilmicosin. *Fodor, L., Varga, J., Gallowitsch, F., Horváth-Papp, I., Miklós, G., Lajcsák, A.* and *Harmath, A.* 41
Comparative pathogenicity study of *Leptospira interrogans* serovar *pomona* strains. *Nagy, Gy.* 315

Epizootiology

Hungary remains free of scrapie and bovine spongiform encephalopathy (BSE). *Kluge, J. P.* and *Glávits, R.* 325

Genetics

Influence of parental origin, litter size and sex on the frequency of splayleg in piglets: A case report. *Tomko, M.* 329

Immunology

T-cell deficiency in Suffolk lambs. *Zomborszky, Z., Horn, É., Tuboly, S., Megyeri, Z., Tilly, P.* and *Szabó, Cs.* 427

Parasitology

Recent achievements in the chemotherapy of myxosporean infections of fish. *Molnár, K.* .. 51
Light and electron microscopic studies on the development of *Sphaerospora colomani* Baska, 1990 and *Chloromyxum inexpectatum* Baska, 1990. *Baska, F.* 59
Sphaerospora siluri n. sp. (*Myxosporea: Sphaerosporidae*) in the kidney of the sheatfish (*Silurus glanis*). *Molnár, K.* 341
Effect of decreased oxygen content on eels (*Anguilla anguilla*) infected by *Anguillicola crassus* (Nematoda: Dracuncoloidea). *Molnár, K.* 349
Efficacy of Butalex in field cases of bovine theileriosis — Short communication. *Mishra, A. K., Sharma, N. N.* and *Viswanathan, C. B.* 361

Pathology

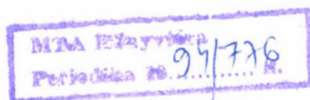
Mycoplasmal arthritis and meningitis in calves. *Stipkovits, L., Rády, Marietta* and *Glávits, R.* 73
Pathomorphological and immunohistological changes in lymph nodes of cows spontaneously infected by bovine leukaemia virus. *Levkut, M., Plank, L., Levkutova, M.* and *Konrád, V.* 89

Pathophysiology

Contribution to the pathobiochemistry of furazolidone-induced oxidative toxicity in chickens. *Sas, B.* 103
Effects of concurrent administration of lead and selenium on some haematological and biochemical parameters of broiler chickens. *Khan, M.Z., Szarek, J., Krasnodębska-Depta, Anna* and *Koncicki, A.* 123
Biochemical changes of the milk in experimental caprine mastitis induced by *Mycoplasma* serogroup 11 (2-D). *Rana, J. S., Gupta, P.P.* and *Ahuja, S.P.* 139
Concurrent oral administration of monensin and selenium to broiler chickens: Effects on concentration of different elements in the liver. *Khan, M.Z., Szarek, J.* and *Markiewicz, K.* 365

Physiology

Clinical biochemical determinations in the Mangalarga-Paulista horse: Reference values. *Novelli, Ethel L.B., Rodrigues, N.L.* and *Chiacchio, S.B.* 151
Thyroid hormone deiodination in the brain of young chickens acutely adapts to changes in thyroid status. *Rudas, P., Bartha, T.* and *Frenyó, V. L.* 381
Thyroxine and triiodothyronine uptake by the brain of chickens. *Rudas, P.* and *Bartha, T.* 395


MTA Könyvtára Periodika 94/776

Toxicology

Biochemical examination of muscle samples from pheasant embryos affected by Wofatox 50 EC (50% parathion-methyl). *Várnagy, L.* 409

Studies on the toxic and immunosuppressive effects of cadmium on the common carp. *Sövényi, J.* and *Szakolczai, J.* 415

Virology

Restriction endonuclease analysis of Hungarian bovine herpesvirus isolates from different clinical forms of IBR, IPV and encephalitis. *Magyar, G., Tanyi, J., Hornyák, Á.* and *Bartha, A.* 159

Serological survey for type D retroviruses and simian immunodeficiency virus in monkeys living in Hungary. *Gyuris, Ágnes, Vajda, G.* and *Földes, I.* 171

Detection of bovine herpesvirus 1 with various types of DNA probes. *Vilcek, Š., Deliová, Iveta, Forgáč, O., Strojný, L., Takácsová, Ildikó, Harvan, M.* and *Benko, G.* 179

Announcement 191

Book reviews 437

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