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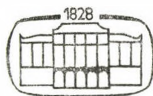

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HISTOMORPHOLOGY OF THE SUBCOMMISSURAL ORGAN IN BUFFALO *(BUBALUS BUBALIS)*

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To study the histomorphology of the buffalo subcommissural organ (SCO), six male buffaloes aged between 1 and 3 years were used. The buffalo SCO histologically consists of, in addition to the ependyma and hypendyma, a well-developed subependymal glial zone. The ependyma of tall columnar ciliated cells with appearance of pseudostratification measures highest in the pars subcommissuralis and lowest in the pars supracommissuralis. The hypendyma is the thickest in the pars retrocommissuralis. The hypendyma consists of cell aggregations in the form of cords, follicles, ducts and segregated cell masses. Supra- and infranuclear vacuolations are common in both the ependymal and hypendymal cells. No evidence of hypendymal ducts opening onto the ventricular surface of the organ was found. Abundance of capillaries, their close association with hypendymal cells and glial fibres around, and the presence of secretory substance intracellularly and in close proximity to the capillaries evidence the endocrine nature of the buffalo SCO.

Keywords. Subcommissural organ, buffalo *(Bubalus bubalis)*, histomorphology.

Systematic histomorphology of the subcommissural organ (SCO) has been reported in various domestic animals (Talanti, 1958; Barlow et al., 1967), rodents (Leonieni, 1968) and in man (Mollgard, 1972). The literature, however, lacks any knowledge on the morphology of the SCO in buffalo. The present study was undertaken to fill this gap.

Materials and methods

The brain tissues including the region of SCO were collected from 6 male buffaloes (aged between 1 and 3 years) immediately after they were killed by bleeding through the carotid artery. The tissues were fixed from 3 animals in 10% buffered neutral formalin, from 2 in Bouin-Dobosque's fixative and from 1 in Baker's formol calcium. The tissues were processed by routine techniques for paraffin blocks using a cedar-oil schedule. The blocks containing two of the neutral formalin-fixed specimens were sectioned in sagittal planes and those containing the rest of the four were sectioned in transverse planes, all at 10 μm thickness serially. The sections at regular intervals were stained with haematoxylin and eosin, Holzer's method for glial fibres and Bergmann's

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modified chrome alum haematoxylin (CAH) method for neurosecretory substance (Humason, 1979).

The thickness of different zones in various regions of the SCO were measured by micrometry at 10 random sites in each region and the average values were recorded for each animal. The means and the ranges are represented in Table I.

Table I

Thickness of ependyma and hypendyma in different parts of the buffalo SCO (in μm)

Parts	Ependyma		Hypendyma	
	Mean	Range	Mean	Range
Pars supracommissuralis	78	52–111	204	75–480
Pars precommissuralis	88	56–126	201	60–130
Pars subcommissuralis	100	67–525	242	75–126
Pars retrocommissuralis	88	56–126	569	225–930

Results

The buffalo SCO was found histologically divisible into three distinct zones, viz. ependyma, subependymal glial zone and hypendyma. The thickness of the ependyma and the hypendyma (the latter including the glial zone) in different regions of the SCO is shown in Table I.

Ependyma. The ependyma consisted of modified epithelial cells. The epithelium appeared stratified tall columnar ciliated with oval vesicular nuclei located basally at varying levels giving an appearance of pseudostratification (Fig. 1). The transition of this layer into the usual ventricular ependyma was found abrupt laterally (Figs 2, 4 and 5) but gradual at its rostral and caudal ends. The thickness of the ependyma varied from 52 to 525 μm, being the highest at the pars subcommissuralis and the lowest at the pars supracommissuralis (Table I).

Numerous crypt-like invaginations limited to the ependyma were evident in young buffalo calves (Figs 2 and 3). These invaginations were more numerous at the pars supracommissuralis, pars precommissuralis and at the bases of the median and lateral longitudinal grooves. In adult buffalo, the invaginations were inconspicuous.

The oval-shaped nuclei of the ependymal cells had well-dispersed chromatin and one or two distinct nucleoli (Figs 1 and 12). Fine eosinophilic granules concentrated towards the ventricular surface and in basal processes close to the underlying blood vessels. Cytoplasmic vacuolations were frequent in the supra- and infranuclear regions (Fig. 1).

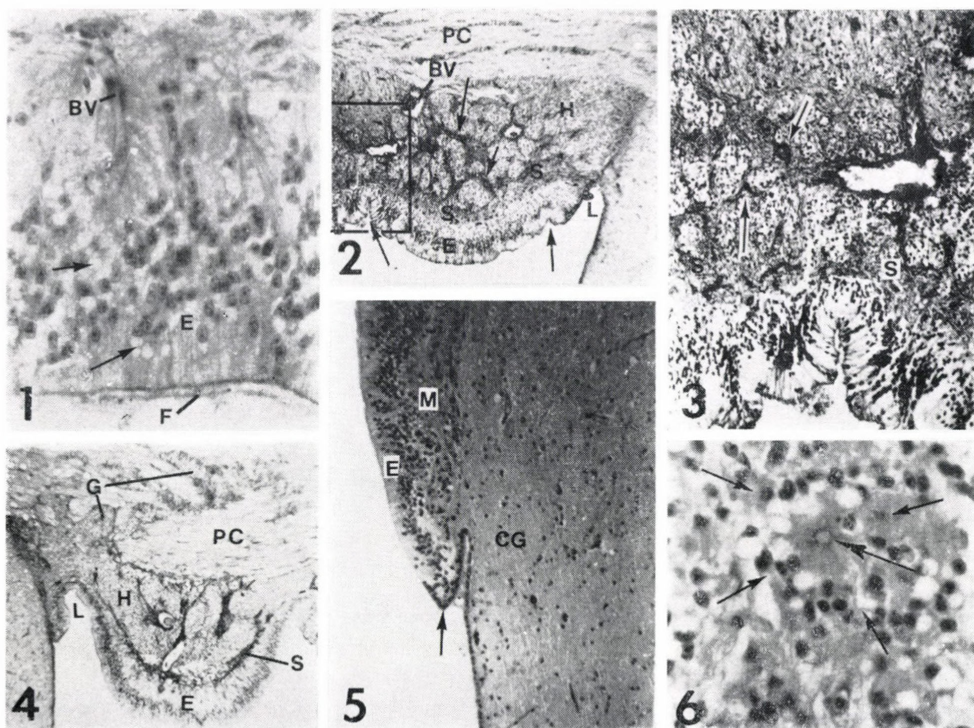

Plate I

Fig. 1. Buffalo calf SCO ependyma (E) showing apical fuzzy layer (F), cell nuclei located at various levels, basal processes terminating around the underlying blood vessels (BV) and vacuolations (arrows). H–E., ×400

Fig. 2. Buffalo calf SCO in cross section through subcommissural region showing three distinct zones: ependyma (E) with crypt-like invaginations (arrows from the ventricular side), lateral longitudinal groove (L), subependymal glial zone (S) and hypendyma (H). Mid-longitudinally running blood vessels (BV), glial trabeculae (arrows) separating hypendymal cell groups, and the posterior commissure (PC) are evident. Holzer's method, ×32

Fig. 3. The area in rectangle in Fig. 2 enlarged, showing glial tissue in the subependymal zone (S) and the glial trabeculae with blood vessels (arrows) extending between hypendymal cell groups. Holzer's method, ×100

Fig. 4. Buffalo calf SCO in cross section through precommissural region showing distinct subependymal zone (S) separating the ependyma (E) from hypendyma (H), the hypendymal glandular tissue (G) extending into the posterior commissure (PC), and lateral longitudinal groove (L). Holzer's method, ×32

Fig. 5. Buffalo calf SCO showing abrupt transition of modified ependyma (E) into usual ventricular ependyma at arrow, and the mixed zone (M) of fewer hypendymal cells with the central gray substance (CG) of the brain. H–E., ×100

Fig. 6. Buffalo calf SCO hypendyma in the subcommissural region showing a hypendymal duct (bounded by thin arrows) lined with pyramidal-shaped cells having large vacuolations and granular cytoplasm staining intensely eosinophilic apically (thick arrow). Other hypendymal cells also show vacuoles. H–E., ×400

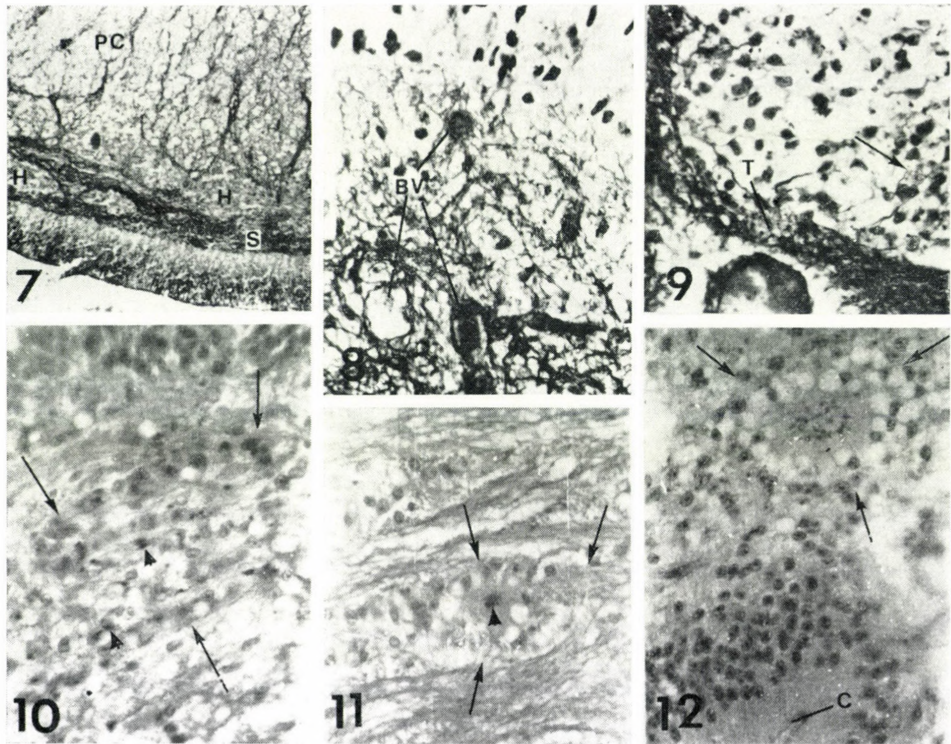

Plate II

Fig. 7. Buffalo calf SCO in mid-sagittal section showing the glial fibres extending from the subependymal glial zone (S) through the hypendyma (H) continuing into the posterior commissure (PC). Holzer's method, ×100

Fig. 8. Buffalo calf SCO showing glial fibre network in the subependymal zone around the longitudinally running blood vessels (BV). Holzer's method, ×400

Fig. 9. Buffalo calf SCO showing a glial trabecula (T) from which the glial fibres traverse in between hypendymal cells (arrow). Holzer's method, ×400

Fig. 10. Buffalo calf SCO showing longitudinally sectioned hypendymal tubules and cords (arrows). CAH-positive secretory granules (arrow heads) and vacuoles are seen in the hypendymal cells. Bergmann's chrome alum haematoxylin (CAH) method for secretory substance, ×400

Fig. 11. Buffalo calf SCO showing a hypendymal duct (bounded by arrows) with secretory substance (arrow head) deep in between the nerve fascicles of the posterior commissure. Bergmann's CAH method for secretory substance, ×400

Fig. 12. Buffalo calf SCO from supracommissural region showing a relatively large hypendymal duct with secretory granules in its cells apically (bounded by arrows). Ependymal crypt (C) is partly seen. Bergmann's CAH method for secretory substance, ×400

The ventricular surface of the ependyma presented a distinct fuzzy layer (Fig. 1). A heterogeneous mass with RBCs and cell debris was seen adhering to this surface, particularly in the median and lateral longitudinal grooves and in the recessus mesocoelicus. The fine granular cytoplasm did not stain with CAH. No evidence of Reissner's fibre on the ventricular surface was noticed.

Subependymal glial zone. Immediately under the ependyma, there was a well-developed pale-staining zone extending throughout the length and width of the SCO, except that in the pars retrocommissuralis it became indistinct and gradually disappeared towards the termination of the organ. This zone chiefly comprised glial tissue (Figs 2, 3, 4 and 7) through which abundant blood vessels traversed longitudinally (Fig. 8). The smaller blood vessels and capillaries arising from this zone were surrounded by the basal processes of the ependymal cells (Fig. 1), which occasionally showed granules of secretory substance perivascularly. A few glial fibres were seen extending into the interstices of ependymal cell processes, and also around the blood capillaries. This zone was generally devoid of characteristic hypendymal cells.

Hypendyma. It was a dense cellular zone between subependymal glial zone and posterior commissure (PC), extending from the beginning of the pars supracommissuralis to the termination of the pars retrocommissuralis. The thickness of this zone varied from 60 to 930 μm, being maximum in the pars retrocommissuralis and minimum in the pars precommissuralis (Table I).

The hypendymal tissue chiefly comprised cell aggregations of different forms separated by a trabecular network of glial tissue along with blood vessels traversing through them (Figs 2–4). Glial fibres were also seen between the cells (Fig. 9). The cell aggregations appeared in forms of cords, follicles and segregated cell masses (Fig. 6). The cellular arrangement seen in rows in sagittal sections indicated some longitudinally running tubular or ductular hypendymal tissue (Fig. 10). No evidence of opening of hypendymal ducts or tubules onto the ventricular surface could be found.

The hypendymal cells were generally irregularly rounded in shape. However, the cells of the hypendymal ducts and follicles were pyramidal, with basally located rounded or oval nuclei (Figs 6 and 12). In other hypendymal cells rounded or oval nuclei were placed centrally or eccentrically. The nuclei appeared vesicular, containing a distinct nucleolus each. Cells with double nucleoli were common. The hypendymal cell cytoplasm stained eosinophilic and frequently was fine granular. Supranuclear and infranuclear vacuolations of variable sizes were common. The cytoplasm of the ductular pyramidal cells particularly close to its narrow lumen contained a deeply eosinophilic granular mass (Fig. 6).

The hypendymal cells in all the forms with similar characteristics were seen extended in between the fascicles of the posterior commissure (Fig. 11).

Hypendymal ducts or follicles of larger sizes with relatively wide lumina were frequent in the pars supracommissuralis (Fig. 12).

Discussion

In rodents, man and domestic animals, the SCO has been described as being divided into two zones, viz. ependyma and hypendyma (Krabbe, 1925; Talanti, 1958; Talanti and Kivalo, 1960; Mollgard, 1972; Mitro and Palkovits, 1981). In the present study on buffalo a distinct zone of glial tissue, a zone that separated the latter from the underlying hypendyma, was observed immediately under the ependyma. For the ease of description this glial zone is described separately as "subependymal glial zone".

Ependyma. In rat, mouse, guinea-pig, hamster, ox, horse, dog, pig, sheep, goat, elk, llama and cow calf, the ependyma was reported to be lined with tall ciliated columnar cells (Wislocki and Leduc, 1952; Talanti, 1958; Talanti and Kivalo, 1960; Isomaki et al., 1965; Kohl, 1975; Mitro and Palkovits, 1981). In buffalo, however, the ependyma consists of modified tall columnar ciliated cells with appearance of pseudostratification, which is in accordance with that reported in sheep (Barlow et al., 1967) and rats (Collins and Woolam, 1979).

The ependyma measured 60 to 80 μm thick below the posterior commissure in rats (Collins and Woolam, 1979). In buffalo, it showed regional variations measuring lowest ependyma, 52–111 μm, in the pars supracommissuralis, gradually increasing to 67–525 μm in the pars subcommissuralis, whereafter again it gradually decreased towards the pars retrocommissuralis.

Goblet-shaped cells with distended apical cytoplasm have been observed in the SCO ependyma of domestic animals (Kolmer, 1921; Talanti, 1958; Talanti and Kivalo, 1960). Kolmer (1921) attributed this to unsatisfactory fixation, while others considered it to be secretory in function. No such goblet-shaped cells were evident in the present study, but supra- and infranuclear cytoplasmic vacuoles were prominent. Sites conforming to the vacuoles stained with Sudan black B could suggest that the vacuoles were due to lipids (Ramkrishna, 1983). This indicates the similarity of its secretory activity with the steroid-secreting type.

Weindl and Schinko (1975) reported in rodents that the mucoid nature of Reissner's fibre had the capacity of binding selective substances, i.e. RBCs and cell debris, at the narrow ventricular system.

In buffalo, however, there was neither any evidence of Reissner's fibre nor that of CAH-positive secretory substance in the ependymal cells and on the fuzzy layer. Having found no Reissner's fibre in the human fetal SCO, Mollgard (1972) stated that the surface coat on it might perform the unknown role played by Reissner's fibre in other species. This is suggestive of the

hypothesis of absorption rather than apical secretion, as well as of the ciliary movement which might have an attractive force to attach the RBCs and cell debris from the CSF in buffalo, thus substituting the function of Reissner's fibre by helping in the cleaning of CSF and/or whatsoever its unknown functions might be in other species.

Subependymal glial zone. In the present study a distinct glial tissue zone, just below the ependyma, was evident throughout the length and width of the SCO, a zone which gradually disappeared at the pars retrocommissuralis. However, in descriptions of other domestic animals this zone has been included in the hypendyma (Krabbe, 1925; Talanti, 1958; Talanti and Kivalo, 1960). It was suggested that the hypendymal glia cells participated in the transport of substances derived from the CSF and also from blood vessels (Cammermeyer, 1965). Because of this capacity, they might serve as "a relay or end station" for some substances passing through the ventricular wall.

Hypendyma. The term "hypendyma" was first initiated by Krabbe (1925) in cattle and dog. It consisted of compact cell islets similar to ependyma, glia cells, neuroglia elements and a few nerve fibres arising from the posterior commissure in ox, horse, dog, pig, cow calf, human fetuses and reindeer (Krabbe, 1925; Talanti, 1958, 1959, 1966; Talanti and Kivalo, 1960; Isomaki et al., 1965; Barlow et al., 1967; Mollgard, 1972). In the present study, the cell aggregations appearing as cell cords with ducts in between and the linear arrangement of cell rows in sagittal sections indicate the presence of tubular glands, which had been seen in ruminants only (Talanti, 1958; Talanti and Kivalo, 1960; Isomaki et al., 1965; Barlow et al., 1967). The opening of the ducts of these tubular glands onto the ependymal surface which had been reported in other ruminants could not be observed in buffalo. The hypendymal ducts were probably not an immediate continuation of crypts of ependyma as these ducts were also visible under the smooth surface ependyma and penetrated even deeply in between the fascicles of the posterior commissure. This speaks in favour of their independent nature.

Olsson (1961) mentioned that the glandular property of the SCO in human fetuses was also seen in the pineal diverticulum, indicating that the posterior lobe of the pineal organ might be built up from this specialized ependyma, so that the secretory activity might be retained for some time in the pineal organ. The same could be true in buffalo, where the hypendymal tissue is well-developed in the wall of the recessus infrapinealis in continuation of the pars supracommissuralis of the SCO.

In bovine fetuses, cylindrical ducts were observed, but not lined with a special cell layer, rather blood vessels entered these ducts (Turkewitsch, 1936). Special tunnel-like cavities were observed in bovine fetuses and reindeer by Talanti (1959, 1966). In the present study, however, no such observations could be made.

The eosinophilia increasing towards the lumen of hypendymal ducts suggested secretory activity similar to that in ependyma, as postulated in ruminants and reindeer (Talanti, 1958, 1966; Talanti and Kivalo, 1960). The supranuclear vacuolations in these hypendymal cell cords have been confirmed to be due to lipids, which might have been removed during processing of paraffin sections (Ramkrishna, 1983). Also osmiophilic lipid bodies have been reported in the hypendymal cells of calves by Isomaki et al. (1965).

In the buffalo SCO, notably no evidence of hypendymal ducts opening into the crypts or onto the surface of ependyma was found. Therefore, the profuse vasculature, close association of blood capillaries with the hypendymal cells and glial fibres, and the presence of glandular CAH-positive secretory substance in the cell masses intracellularly as well as in close proximity of capillaries indicate the endocrine nature of the SCO.

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COMPARATIVE STUDY ON THE QUANTITY OF MINERAL ELEMENTS EXCRETED IN THE FAECES BY THE RABBIT *(ORYCTOLAGUS CUNICULUS DOMESTICUS)* AND WILD RABBIT *(ORYCTOLAGUS CUNICULUS)*

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Eleven faecal samples collected from wild rabbits *(Oryctolagus cuniculus)* living in different regions of Hungary and 10 true (hard) faecal samples collected from rabbits *(O. cuniculus domesticus)* kept in agricultural rabbit farms were examined for 11 mineral elements. The mineral element content of the feedstuffs was also estimated together with their crude protein, crude fat, crude fibre and crude ash contents. The results are summarized in Tables II–V. The most important conclusions are as follow: (1) The average quantity of Ca, P, S, Mg, Na, Zn and Cu was found lower than the reported values, while that of K, Al and Fe was three- to fourfold higher. (2) In the faeces of rabbits the quantity of Ca, P, Na, Fe and Cu, usually administered in the premixes, was higher than in the faeces of wild rabbits. (3) Significant differences were found also in the mineral element contents of the 9 feeds; namely, the difference between the maximal and minimal concentrations of Ca, P, S, Fe, Mn and Zn was twofold, while in the case of Na, the difference was threefold. (4) The quantity of mineral elements, except for P, Mn and Na, was two- to eightfold higher than required in all the examined feeds, which was deleterious from both veterinary hygienic and economic points of view. (5) Except for K and Na, the average quantity of mineral elements was two- to fourfold higher in the faeces, indicating an enrichment and excretion of the mineral elements taken up in excess. (6) The lower quantities of K and Na measured in the faeces suggest that their excess is excreted in the urine also in the rabbit. Further studies are required to throw light on how close the correlation is between the quantity of mineral elements taken up in the feeds and excreted in the faeces.

Keywords. Comparative study, mineral elements, faeces, wild rabbit *(Oryctolagus cuniculus)*, rabbit *(Oryctolagus cuniculus domesticus)*.

The increasing demand for table rabbit on a world scale resulted in a dynamic development of rabbit keeping and breeding during the past two decades. Simultaneously, besides dealing with the improvement of breeds and management technology, a very active research work has started on the nutrition of the rabbit. Remarkable results have been achieved in the determination of nourishing substance, crude fibre and drinking-water requirements of rabbits, as well as in the specification of composition and optimal physical form of complex rabbit feeds (Schmidt, 1960; Holdas, 1968; Kolb and Gürtler, 1971; Holdas et al., 1975; Scheelje et al., 1975; Jeroch, 1976; Lebas, 1975; Csikváry, 1982; Robbins, 1983; Fekete and Bokori, 1984).

Information was lacking, however, on the mineral-element and vitamin metabolism and requirements of rabbits. As to the mineral elements, the majority of data are related to the metabolism of Ca, P and Na (Scoggins et al., 1970; Holdas et al., 1975; Jeroch, 1976; Underwood, 1977; Robbins, 1983)

and to the related animal-health problems (Fore and Morton, 1982; Kötsche and Gottschalk, 1977; Robbins, 1983). The literary data on the mineral element requirements of rabbits are inconsistent (Table I), and few data are available on the mineral element content of faeces. There are some references on the mineral element composition of faeces (Fekete and Bokori, 1978; Holdas, 1979; Csikváry, 1982; Robbins, 1983) and on the low, gradually decreasing (from 10 μg to 4 μg/ml) Fe content of milk during lactation (Scoggins et al., 1970; Nagy et al., 1976). Very few reports deal with the excretion of mineral elements in the urine (Robbins, 1983) and with the average ash and mineral element contents of rabbit faeces (Fekete and Bokori, 1984; Fekete, 1984). Under regular feeding conditions, Fekete (1984) found the following average quantities of mineral elements in the faeces of the rabbit: K = 3.3; Ca = 26.1; P = 16.3; S = 3.6; Mg = 5.8; Na = 2.6 g/kg; while Al = 178; Fe = 420; Mn = 302; Zn = 420; Cu = 43; B = 8.9; and Mo = 1.33 mg/kg. The quantity of crude ash was given as 13.7 $\pm$ 2.5% (Fekete and Bokori, 1984).

The present study had two objectives. On the one hand, we wished to collect data on the quantity of major mineral elements in the faeces of the wild rabbit *(Oryctolagus cuniculus)* living under natural conditions and of the rabbit *(Oryctolagus cuniculus domesticus)* consuming known feedstuffs, and looked for differences between the mineral-element composition of the faeces of these two rabbit groups. On the other hand, we searched a possible correlation between the quantity of mineral elements consumed in the feed and excreted in the faeces. The latter correlation, if exists, deserves attention because it may facilitate the monitoring of the mineral element supply in rabbits, similarly to other species (Bokori and Tölgyesi, 1981).

Materials and methods

The study was carried out on faecal samples collected from wild rabbits in different regions of Hungary during the first half of October, and on 6 (2 broiler, 4 breeding) and 4 faecal samples collected in 4 large-scale and 4 backyard rabbit farms, respectively, in November. The mineral element content of the faecal samples and of the feeds consumed by the rabbits was also estimated. For the analytical examinations, specimens of fresh, true (hard) faeces, voided a few hours before collection, were used. The samples were dried up to weight-balance and stored at +4 °C until tested. The nutritional data (crude protein, crude fat, crude fibre, crude ash and nitrogen-free extract) were examined according to the Hungarian standard MSz-6830. The mineral elements were determined after wet destruction of 2 g of faecal samples partly in a Perkin-Elmer 5000 type atom-absorption apparatus (K, Mg, Ca, Na, Mn, Zn), partly by colorimetric methods (P, Al, Fe, Cu). S was determined by turbidimetry.

Table I

Mineral substance requirement of rabbits as reported by various authors

Authors	K	Ca	P	Mg	NaCl	Fe	Cu	Mn	Zn	Co	J
	in % of dry matter in the feed					mg/kg dry matter in the feed					
Holdas, Csikváry, Szikora (1975)	—	8–10	0.6–0.7	0.3–0.4	0.3–0.5	50–60	4.0–5.0	—	20–25	0.2–0.3	0.2–0.3
Scheelje et al. (1975)	—	0.5–1.2	0.5–0.8	—	—						
Jeroch (1976)	—	—	—	0.4	—	35	5.0	40	40	1.0	—
N.R.C. (1977)											
Growing rabbits	0.6	0.40	0.22	0.3–0.4	0.2	—	3.0	8.5	—	—	0.2
Pregnant rabbits	0.6	0.45	0.37	0.3–0.4	0.2	—	3.0	2.5	—	—	0.2
Lactating rabbits	0.6	0.75	0.50	0.3–0.4	0.2	—	3.0	2.5	—	—	0.2
Csikváry (1982)		0.8–1.0	0.6–0.7	0.3–0.7	1.0	50–60	4.0–5.0	—	20–25	0.2–0.3	0.2–0.3

Table II

Mineral element contents in the faeces of wild rabbits *(Oryctolagus cuniculus)*

Specimen no.	K	Ca	P	S	Mg	Na	Al	Fe	Mn	Zn	Cu
	g/kg						mg/kg				
1	18.0	12.7	6.3	3.8	3.2	1.1	399	984	634	203.8	14.5
2	20.1	11.5	8.6	4.0	3.4	0.9	350	886	542	210.1	17.1
3	16.3	24.2	4.7	3.6	3.4	1.2	330	902	610	240.8	12.2
4	17.1	20.3	4.4	4.5	5.1	2.9	441	753	126	124.6	14.9
5	12.8	19.4	4.9	5.1	5.3	1.8	610	804	98	131.0	15.7
6	10.8	22.6	5.0	3.1	4.1	1.4	665	1106	90	133.8	19.4
7	13.5	10.0	4.2	2.9	2.7	1.2	607	874	318	130.1	13.0
8	17.0	10.2	3.7	2.2	2.6	0.9	1010	1150	130	125.0	12.0
9	25.3	15.2	5.9	2.7	3.9	1.7	520	748	250	104.0	15.0
10	22.0	17.1	4.9	2.1	4.4	1.1	677	903	310	150.8	12.1
11	19.6	16.4	4.8	3.0	3.8	0.9	512	560	278	121.0	11.2

Table III

Nutritional data of the faeces of rabbits (in per cents)

Farm no.	Crude ash	Crude protein	Crude fat	Crude fibre	N-free extract
1 Large-scale farm					
Broiler rabbit	10.13	18.58	2.37	37.81	27.41
Breeding rabbit	14.97	17.67	3.06	36.48	25.62
2 Large-scale farm					
Broiler rabbit	10.02	18.48	2.17	34.59	31.54
Breeding rabbit	9.90	13.99	3.25	36.73	33.73
3 Large-scale farm					
Breeding rabbit	14.17	19.67	1.78	35.89	26.29
4 Large-scale farm					
Breeding rabbit	8.52	17.12	2.46	37.17	31.43
5 Backyard farm					
Breeding rabbit	9.22	20.15	2.56	36.64	28.63
6 Backyard farm					
Breeding rabbit	9.54	13.94	3.05	34.95	37.03
7 Backyard farm					
Breeding rabbit	8.61	18.18	3.15	36.87	31.39
8 Backyard farm					
Breeding rabbit	15.46	16.02	2.76	33.89	30.07

Results

The mineral element contents of 11 faecal samples collected from wild rabbits are shown in Table II. Nutritional data and mineral element contents of the faecal samples collected from rabbits and those of the feeds are shown in Tables III and IV. Mean values for the mineral element contents of faecal samples collected from both groups, standard deviations and the variation coefficients are to be found in Table V.

Conclusions and discussion

As shown in Table V, the average quantities of mineral elements found in the faeces of rabbits were as follow: K = 13.4; Ca = 19.3; P = 9.1; S = 4.4; Mg = 4.7; Na = 2.1 g/kg; Al = 400; Fe = 1145; Mn = 218; Zn = 164; Cu = 30 mg/kg of dry matter. The same values in the faeces of wild rabbits were the following: K = 17.5; Ca = 15.4; P = 5.2; S = 3.4; Mg = 3.8; Na = 1.4 g/kg; Al = 556; Fe = 879; Mn = 328; Zn = 152; Cu = 14 mg/kg of dry matter. The measured average quantities for Ca, P, S, Mg, Na, Mn, Zn and Cu

were lower to a varying extent, while the values for K, Al and Fe were significantly higher than those reported by Fekete (1984) for the mineral element content of faeces, collected from rabbits fed a rabbit feed prepared from the usual components. The average percentage of crude ash (13.8%) was comparable.

Comparison of the average mineral element concentrations in the faeces of rabbits and wild rabbits has shown that the quantity of Ca, P, Na, Fe and Cu, administered first of all in the premixes, was always higher in the faeces of rabbits. Mineral elements showed a great variability in the faeces of rabbits kept either in large-scale or backyard farms, depending on the mineral element content of consumed feeds (see below) and on the varying quantity of other fodders consumed. Standard deviations of the quantities of mineral elements in the faeces of free-living rabbits have indicated that they consumed different feedstuffs with different mineral element contents in each region of Hungary. On the other hand, the standard deviations for mineral element concentrations, except for Mn, were generally lower in the case of wild rabbits.

Significant differences were found among the mineral element contents of the 9 rabbit feeds. The difference between the maximal and minimal concentrations was twofold in the case of Ca, P, S, Al, Fe, Mn and Zn, and threefold in the case of Na, indicating significant differences in the production and composition of rabbit feeds in the farms.

Comparing the average quantities of mineral elements measured in the feeds with the data of mineral element requirements for rabbits (Table I), we can conclude that the quantity of mineral elements, except for P, Mn and Na, and especially that of some microelements (Fe, Mn, Zn) were two- to eightfold higher than required. This fact worsens the profitability of table rabbit production on the one hand and disturbs the utilization of other mineral elements on the other. Moreover, the excretion of the excess loads the organism.

Comparing the mineral element content of the ad libitum consumed feed and that of the faecal samples only, the following conclusions can be drawn:

(1) The concentration of the investigated elements, except for K and Na, was generally two- to fourfold higher; i.e., they were taken up in excess and, after enrichment in the faeces, they were excreted. In this respect, faecal samples collected from rabbits consuming also meadow hay with high K, Ca and P concentrations on farm no. 8 proved to be an exception.

(2) Smaller quantities of K and Na were measured in the faecal samples, indicating that the excess of these elements (mainly of K in this experiment) was excreted in the urine by rabbits as well.

(3) Further experiments are required to throw light on how close the correlation is between the quantity of individual mineral elements taken up in the feed and excreted in the faeces, and on the usefulness of this correlation for monitoring the mineral element supply of rabbits.

Table IV

Mineral elements in feeds and

Farm no.	Feed	Mineral elements K	Ca	P
				g/kg of
1 Large-scale farm				
	Rabbit feed	11.3	12.7	4.1
	Alfalfa hay	19.6	19.5	2.4
Broiler rabbit	Faeces	6.7	19.5	8.6
Breeding rabbit	Faeces	25.5	20.5	6.2
2 Large-scale farm				
	Rabbit feed	14.7	12.6	7.0
Broiler rabbit	Faeces	6.6	20.2	12.5
	Rabbit feed	13.1	9.2	4.8
Breeding rabbit	Faeces	6.6	13.3	8.7
3 Large-scale farm				
	Rabbit feed	15.1	13.2	4.9
Breeding rabbit	Faeces	13.6	31.6	14.5
4 Large-scale farm				
	Rabbit feed	12.6	11.8	4.9
Breeding rabbit	Alfalfa hay	27.6	20.6	2.7
	Faeces	9.7	16.0	8.2
5 Backyard farm				
	Rabbit feed	13.7	11.2	5.9
Breeding rabbit	Faeces	8.6	16.3	6.9
6 Backyard farm				
	Rabbit feed	14.2	18.8	4.6
Breeding rabbit	Bran	5.8	1.1	3.0
	Faeces	14.0	10.2	6.5
7 Backyard farm				
	Rabbit feed	16.5	14.7	5.0
Breeding rabbit	Alfalfa hay	12.5	22.7	1.9
	Faeces	12.6	20.0	8.4
8 Backyard farm				
	Rabbit feed	12.0	12.7	5.3
Breeding rabbit	Meadow + alfalfa hay	39.0	71.1	2.9
	Faeces	30.6	25.5	10.5

Table V

Statistical indices of mineral element contents in the faecal specimens collected from rabbits and wild rabbits

		K	Ca	P	S	Mg	Na	Al	Fe	Mn	Zn	Cu
		g/kg dry matter						mg/kg dry matter				
Rabbit	$\bar{x}$	13.4	19.3	9.1	4.4	4.7	2.1	400	1145	218	164.8	30.3
	s	8.3	6.1	2.7	2.2	0.8	0.8	64	299	15	23.6	10.7
	CV	61.6	31.4	29.2	50.6	16.0	36.0	16.0	26.1	6.8	14.3	35.3
Wild rabbit	$\bar{x}$	17.5	15.4	5.2	3.4	3.8	1.4	556	879	328	152.3	14.3
	s	4.2	4.2	1.3	0.9	0.9	0.6	193	167	195	44.7	2.5
	CV	24.0	27.3	12.0	27.6	23.0	42.0	34.7	18.9	59.4	29.3	17.5

faecal specimens of rabbits

Mineral elements							
S	Mg	Na	Al	Fe	Mn	Zn	Cu
dry matter			mg/kg of dry matter				
2.9	2.3	2.2	442	578	92	68.8	10.5
2.4	3.4	3.2	514	550	68	31.1	8.4
4.2	4.0	1.9	459	1598	224	184.6	25.0
5.3	5.3	3.9	416	1551	216	152.5	24.8
3.0	2.9	3.0	185	319	98	109.9	12.1
2.9	3.5	1.7	348	973	200	173.6	26.5
2.5	2.4	3.0	187	273	61	73.6	11.2
2.8	5.0	1.6	495	1339	280	190.1	42.2
2.3	2.8	2.9	253	302	95	98.2	14.5
4.4	5.4	2.6	314	1198	236	181.2	53.2
3.0	2.9	2.0	308	421	84	72.1	9.8
3.3	3.4	0.5	459	407	54	22.3	5.3
2.1	5.8	1.6	382	1198	245	158.0	33.1
2.3	2.5	2.9	223	307	80	97.7	11.0
3.8	4.4	1.8	357	1001	218	180.9	30.7
4.3	3.4	3.9	240	326	82	85.4	11.8
1.1	1.3	0.1	17	44	21	21.4	2.3
3.8	4.0	1.7	493	599	120	143.0	14.7
4.0	3.0	3.1	185	324	102	54.7	10.6
4.3	3.1	1.1	348	575	31	25.1	4.8
4.6	4.6	1.5	342	972	225	171.0	28.2
4.9	2.6	1.7	183	311	98	65.2	8.0
4.0	2.4	0.4	568	471	37	26.5	4.9
10.1	5.4	2.9	391	1022	216	11.3	24.6

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ANTINUTRITIVE EFFECT OF DIFFERENT LUPIN *(LUPINUS)* SPECIES ON THE PROTEIN METABOLISM OF RATS

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Short-term and long-term feeding experiments were conducted with growing, male, white Wistar rats to get an insight into the antinutritive effect of lupin alkaloids.

A short-time feeding of diets of high (0.2%) alkaloid content to rats significantly ($P < 0.001$) impaired protein utilization. The utilization of protein was improved by giving synthetic methionine. If the same was fed for prolonged periods, lack of appetite, loss of body mass, and deaths occurred. Lipid droplets appeared in the liver tissue. The amino-acid composition of the hepatic protein underwent a change; especially sulphur-bearing amino acids (cystine and methionine) decreased in quantity.

If the mixed feed containing lupin seeds had a lower alkaloid content (about 0.1%) and, completed with synthetic (DL) amino acids, was fed for a long period, protein utilization and body mass gain was significantly ($P < 0.01$) worse than those for the control group fed a diet free from lupin seed. A further decrease of the alkaloid content (to about 0.01%) resulted in more favourable protein utilization and body mass gain; however, the values of the physiological parameters still failed to reach those measured for the control animals.

Keywords. Lupin (*Lupinus* spp.), alkaloid, antinutritive effect, protein metabolism, protein utilization, body mass gain, rat.

Lupin seeds contain considerable quantities of biologically valuable proteins which can be good-quality protein sources in the feed of monogastric animals. According to Bódis (1983), the average yields of lupin species can be further increased on the basis of their genetic capacity. However, the use of lupin seeds for feeding purposes is restricted by the presence of substances of alkaloid nature, known to possess an antinutritive effect. Alkaloids are detectable in all lupin seeds (Bohlmann and Schumann, 1967; Schoeneberger et al., 1982; Bélteky and Kovács, 1982); however, their quantity and composition vary widely. As a rule, the seeds of sweet lupin species contain less, whereas those of bitter lupin species more, alkaloid. According to Wittenburg and Nehring (1967), Richter and Schiller (1956) and Szerafin (1984), in lupin species the quantity of alkaloids may reach 2 to 3%. The alkaloids of lupin species, collectively termed lupin alkaloids (e.g. sparteine, lupinidine, lupanine, hydroxylupanine, lupinine, etc.) slightly differ in their chemical structure, and act as inhibitors of the central nervous system. The symptoms of acute toxicosis include (Garner, 1957) inappetence, tympania, fever, dyspnoea, tachycardia, symptoms which may finally lead to respiratory arrest and death. The symptoms of chronic toxicosis differ by species, but usually include retarded growth, inappetence, loss of body mass, liver damage, etc.

To obtain a more thorough knowledge of the antinutritive action exerted by lupin seed alkaloids, feeding experiments were conducted with Wistar rats. The aim was to demonstrate the changes produced in the rats' protein metabolism by feeding seeds of the white, bitter lupin "Vajai" rich in alkaloids, those of the white, sweet lupin "Nyírségi" of lower alkaloid content, and those of the yellow, sweet lupin called "Borluta". A further object was to determine whether such metabolic changes can be prevented by supplementing the diet with DL-amino acids or with proteins of other origin.

Materials and methods

Two experiments (experiments A and B) were conducted with growing, male, white Wistar rats.

In experiment A the animals were accommodated either singly or in groups.

The individually kept rats (3 groups, each consisting of 6 rats) were used in a 10-day study of nitrogen metabolism. The following parameters were determined: biological value (BV) of the proteins taken up in the feed; total digestibility (TD) and apparent digestibility (AD) of the protein; net protein utilization (NPU) and productive protein utilization (PPU; Bock et al., 1964; Eggum, 1973).

In experiment A the rats kept in 3 groups, each containing 10 animals, received the same diet as the animals housed individually, for a period of 21 days.

In the diet fed to Group 1 (control; C) soybean meal, whereas in the diets of Groups 2 and 3 the white, bitter lupin called "Vajai" was the sole protein source. The diet fed to rats of Group 3 was supplemented with 0.3% DL-methionine (Table I).

In experiment B 7 groups, each comprising 10 rats, were formed. The feeding trial lasted 60 days. The diets were formulated so that the soybean protein representing the main protein source was replaced in 50% (Groups 2, 4 and 6) or in 30% (Groups 3, 5 and 7) with proteins of different lupin seeds. The diets fed to these groups were supplemented with DL-lysine and DL-methionine (Table II).

At the end of the experiment, blood and liver samples were taken from the rats kept in groups.

The total protein N and total amino-acid N contents were determined in the blood of rats of both experiments A and B, by the method described by Bálint (1962). Blood urea content was measured using the Berthelot reaction (Klinisches Laborb. Merck, 1974). The liver samples were examined histologically, and their protein, amino acid and alkaloid contents were determined.

Table I

Composition, protein, amino acid and alkaloid contents of diets formulated with soybean and "Vajai" white, bitter lupin (in %)

Feed	Groups		
	1 (C)	2	3
Extracted soybean	22.1	—	—
"Vajai" white, bitter lupin	—	26.2	26.2
Starch	52.9	48.8	48.5
Oil	10.0	10.0	10.0
Sugar	10.0	10.0	10.0
Vitamin premix	1.0	1.0	1.0
Mineral premix	4.0	4.0	4.0
DL-methionine	—	—	0.3
Total:	100.0	100.0	100.0
Crude protein	9.5	9.5	9.5
Cystine	0.16	0.09	0.09
Methionine	0.15	0.09	0.39
Lysine	0.60	0.46	0.46
Total alkaloid	—	0.20	0.20

Table II

Composition of diets formulated with maize, wheat, soybean and different lupin seeds (in %) (Experiment B)

	Groups						
	1 (C)	2	3	4	5	6	7
Maize	41.0	40.0	40.0	41.5	41.0	40.5	41.0
Wheat	40.8	39.7	40.7	39.7	40.7	40.2	40.2
Extracted soybean	13.0	6.5	9.0	6.5	9.0	6.5	9.0
"Vajai" white, bitter lupin	—	8.5	5.0	—	—	—	—
"Nyírségi" white, sweet lupin	—	—	—	—	—	7.5	4.5
"Borluta" yellow, sweet lupin	—	—	—	7.0	4.0	—	—
L-lysine	0.1	0.2	0.2	0.2	0.2	0.2	0.2
DL-methionine	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Vitamin premix	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Mineral premix	4.0	4.0	4.0	4.0	4.0	4.0	4.0
Total:	100.0	100.0	100.0	100.0	100.0	100.0	100.0
Crude protein	15.2	15.2	15.2	15.3	15.3	15.2	15.3
Cystine	0.31	0.30	0.31	0.32	0.31	0.31	0.32
Methionine	0.36	0.34	0.35	0.33	0.34	0.35	0.35
Lysine	0.76	0.80	0.83	0.81	0.84	0.83	0.84
Total alkaloid	—	0.07	0.05	0.005	0.003	0.008	0.005

Table III

Protein, amino acid and alkaloid content of the experimental diets, in dry matter per cent

Parameter	Extracted soybean	Wheat	Maize	"Vajai" white, bitter lupin	"Nyírségi" white, sweet lupin	"Borluta" yellow, sweet lupin
Crude protein	42.9	14.3	9.2	33.7	36.9	42.4
Cystine	0.73	0.34	0.20	0.35	0.59	0.64
Methionine	0.69	0.24	0.22	0.30	0.41	0.30
Lysine	2.78	0.40	0.27	1.75	2.07	2.07
Total alkaloid	—	—	—	0.78	0.09	0.11

The nutrient content of the feeds was determined on the basis of the Hungarian standard MSz 68-30 (1966),* while the amino acids with the BIO-Cal Type 200 amino-acid analyser.

Alkaloid content was measured according to the Hungarian standard MSz 08 (1262-80; 1980).**

Results

The crude protein, amino acid and alkaloid contents of feeds used in experiments A and B, as measured by us, are presented in Table III.

Of the three lupin seeds, "Borluta" had a crude protein content nearly equal to that of soybean (42.4%); "Nyírségi" contained 36.9%, while "Vajai" 33.7% crude protein. The crude protein content of soybean, wheat and maize used in the feeding trials can be considered to be in the range of usual values, on the basis of data shown in Table IV.

The "Vajai" lupin seed contains less cystine (0.35%), methionine (0.30%) and lysine (1.75%) than the two other lupin seeds. The sulphur-bearing amino acid and lysine contents of the soybean, wheat and maize can be considered to be in the range of usual values.

As regards total alkaloid, the "Vajai" is the first (0.78%), followed by "Borluta" (0.11%) and "Nyírségi" (0.09%).

Experiment A

Studies on N metabolism. The protein utilization results determined by N-metabolic studies are shown in Table IV. The animals consuming bitter lupin only (Group 2) had the lowest values of N balance (15 mg/day/animal), the lowest biological value of protein (46.2%), and other protein utilization values were also the lowest. When the lupin "Vajai" was supplemented with

* MSz 68-30 (1966): Determination of the nutritive value of feeds

** MSz 08 (1262-80) (1980): Sweet lupin for industrial feed production

Table IV

Protein utilization data obtained in the feeding trial (Experiment A)

	Groups		
	1 (C)	2	3
N balance, mg/day/animal	54.0	15.0	44.0
Biological value (BV), %	73.1	46.2	69.6
Total digestibility (TD) of protein, %	72.7	78.0	77.9
Apparent digestibility (AD) of protein, %	59.4	65.0	64.9
Net protein utilization (NPU), %	53.0	35.9	54.1
Productive protein utilization (PPU), %	29.8	13.1	29.8

0.3% DL-methionine, the values either reached (PPU: 29.8%; NPU: 54.1%; BV: 69.6%) or approximated those obtained for rats fed extracted soybean (Group C).

Blood analyses. From Table V it is apparent that no appreciable difference existed between the groups in the total protein content of the blood. However, blood urea and total amino-acid N values were lower in Groups 2 and 3 than in Group C (Group 1).

Liver analyses. Histopathologically, no lesions were found in the rats of Group C (1), while in those of Groups 2 and 3 the RHS cells of the liver contained lipid granules.

The protein content of the liver was 73.3, 70.9 and 72.2% for Groups C (1), 2 and 3, respectively.

As regards the amino-acid composition of liver proteins (Table VI), with the exception of aspartic acid and glutamic acid, all amino acids reached higher values in Group C than in the other two groups. In rats of Groups 2 and 3 the amino acid content of the liver proteins was lower than in the control rats. This especially held true for cystine and methionine, but also for alanine, isoleucine, phenylalanine, lysine and histidine.

No alkaloids were detectable in the livers.

Table V

Blood parameters of the rats kept in groups (Experiment A)

	Groups		
	1 (C)	2	3
Total protein, g/l	69.85 ± 5.03	67.30 ± 2.22	67.48 ± 4.65
Urea, mmol/l	7.64 ± 0.70	9.57 ± 0.41	9.34 ± 0.37
Total amino-acid N, mmol/l	7.94 ± 0.43	6.89 ± 0.32	6.45 ± 0.24

Table VI

Amino acid contents of the livers, in dry matter per cent (Experiment A)

Amino acids	Groups		
	1 (C)	2	3
Aspartic acid	5.2	5.6	5.5
Threonine	3.2	3.1	2.6
Serine	3.1	2.9	2.9
Glutamic acid	5.2	6.2	5.7
Proline	4.1	3.2	3.7
Glycine	3.7	2.6	2.7
Alanine	5.9	3.6	4.1
Cystine	1.8	1.2	1.5
Valine	3.8	3.5	3.5
Methionine	2.3	1.1	1.5
Isoleucine	3.1	2.6	2.7
Leucine	7.2	6.1	6.1
Tyrosine	2.3	2.2	2.2
Phenylalanine	3.4	2.6	2.2
Lysine	6.3	5.2	5.1
Histidine	1.6	1.3	1.1
Arginine	6.2	6.2	6.6

Experiment B

Table II shows that the diet fed to rats of Group C (1) contained exclusively soybean meal as protein source, besides maize and wheat. This diet was supplemented with lysine and methionine. To the diets of Groups 2 and 3 "Vajai", to that fed to Groups 4 and 5 "Nyírségi", while to those of Groups 6 and 7 "Borluta" lupin was added in a quantity to replace 50 and 30% of the soybean protein, respectively. Thus, the crude protein content of the diets was practically identical; their amino acid contents were adjusted to the same level by adding DL-lysine and DL-methionine.

Depending on the relative amounts and alkaloid contents of the different lupin seeds, the diet of Group 2 was the most abundant in alkaloids (0.07%), followed by the diets of Groups 3, 6, 4, 7 and 5, containing 0.05, 0.008, 0.005, 0.005 and 0.003% alkaloid, respectively.

Table VII

Blood parameters of (Experi-

	Groups		
	1 (C)	2	3
Total protein, g/l	75.4 ± 4.9	72.3 ± 2.3	70.8 ± 5.8
Urea, mmol/l	5.37 ± 0.71	8.03 ± 0.74	8.05 ± 0.74
Total amino-acid N, mmol/l	8.25 ± 1.13	7.53 ± 0.48	7.10 ± 0.56

Blood analyses. The results are summarized in Table VII. The total protein content of the blood was between 69.8 ± 2.2 and 76.6 ± 1.4 g/l. Blood urea concentration was the lowest in rats of Group 1 (5.37 ± 0.71 mmol/l) and the highest in those of Group 3 (8.05 ± 0.74 mmol/l). As to the total amino-acid N concentration, no significant differences existed between the groups, the extreme values being 7.10 ± 0.56 and 8.25 ± 1.13 mmol/l.

Liver analyses. No histopathological changes were demonstrable in the livers.

The protein content of liver tissue ranged between 74.1 and 76.5%. The liver proteins of the rats fed lupin seeds were poorer in sulphur-bearing amino acids than those of the Group C rats. No appreciable difference existed in the concentration of the other amino acids (Table VIII).

No alkaloids were detectable in the livers.

Discussion

The results of experiment A have shown that both the short-term (10 days) and the long-term (21 days) feeding of "Vajai" lupin seeds, rich in alkaloids, considerably influenced the physiological parameters studied by us, and resulted in a very poor protein utilization (BV = 46.2%; NPU = 35.9%; PPU = 13.1%; Table IV). During the experiment the body mass of the rats decreased by 0.5 g/day on the average. Supplementation of the diet with 0.3% DL-methionine brought about a 20 to 25% improvement in the different protein utilization indices. Thus, the average body mass gain of the rats was 1.0 g. In the case of short-term feeding, methionine supplementation reduced the antinutritive effect of the lupin alkaloids.

Prolonged feeding of a diet of 0.2% alkaloid content significantly ($P < 0.001$) worsened protein utilization, even if the diet was supplemented with 0.3% DL-methionine. Rats of both experimental groups (Groups 2 and 3) showed inappetence, loss of body mass and, occasionally, deaths occurred. Histologically, the RHS cells of the liver contained lipid granules. As to the amino acid composition of the liver proteins, very low concentrations of

the experimental rats
ment B)

Groups			
4	5	6	7
73.6 ± 5.4	71.6 ± 6.2	76.6 ± 1.4	69.8 ± 2.2
6.68 ± 0.79	6.33 ± 0.55	7.14 ± 0.71	6.80 ± 0.66
7.98 ± 0.55	7.44 ± 0.47	7.93 ± 1.19	7.23 ± 1.55

Table VIII

Amino acid contents of livers, g/100 g dry matter (Experiment B)

Amino acids	Groups						
	1 (C)	2	3	4	5	6	7
Aspartic acid	6.1	5.5	5.6	5.2	6.2	5.2	5.8
Threonine	3.8	3.6	3.7	3.7	3.7	3.9	4.0
Serine	3.4	2.6	3.1	3.9	3.2	3.0	2.8
Glutamic acid	4.4	3.3	3.9	7.2	7.6	7.3	8.7
Proline	4.3	3.4	3.9	3.2	4.1	2.3	4.0
Glycine	4.0	3.9	3.9	3.6	3.8	3.7	4.0
Alanine	5.6	4.5	5.3	4.6	4.9	5.5	5.7
Cystine	2.0	1.5	1.7	1.8	1.8	1.7	1.9
Valine	5.5	3.3	5.4	3.6	5.3	4.8	5.5
Methionine	2.9	1.2	2.0	2.4	2.4	2.1	2.6
Isoleucine	3.7	3.3	3.4	2.3	3.9	3.4	2.6
Leucine	9.1	7.5	9.1	7.9	8.6	8.6	9.2
Tyrosine	2.8	2.7	2.8	2.3	2.4	2.6	2.3
Phenylalanine	4.5	3.2	4.2	3.8	3.4	4.4	4.5
Lysine	6.5	6.2	6.5	6.2	6.3	6.1	6.1
Histidine	1.9	1.4	1.5	1.6	1.5	1.4	1.6
Arginine	6.4	4.8	5.1	5.1	5.6	5.7	5.6

sulphur-bearing amino acids were obtained for the experimental rats. In a 30-day feeding trial conducted with rats, Richter and Schiller (1956) fed a diet of 0.2%, or higher, alkaloid content, and observed lack of appetite, loss of body mass, abortions among pregnant rats, and other metabolic disorders.

Our results furnish further evidence that feeding diets of 0.2% alkaloid content to rats markedly worsens protein utilization even if the experimental feeding lasts a short time. Protein utilization can be improved by giving 0.3% DL-methionine. When this diet was fed over a prolonged period (21 days), the rats showed inappetence, loss of body mass and, occasionally, died. The liver tissue showed pathological changes, the amino acid composition of liver proteins underwent a change; especially the sulphur-bearing amino acids decreased in quantity.

In experiment B, where lupin seeds were completed with other feeds (soybean, wheat, maize) and synthetic amino acids, the diet of nearly 0.1% alkaloid content ("Vajai" lupin) brought about a change in the physiological parameters. Diets with less than 0.1% alkaloid content failed to produce appreciable changes in these physiological indices. In the rats fed "Vajai" lupin, blood urea concentration significantly ($P < 0.01$) rose as compared to the control rats, indicating a poorer utilization of the ingested protein. At the same time, in groups of rats consuming sweet lupin seeds with alkaloid contents around 0.01%, changes of such a degree did not occur (Table VII). While the rats fed "Vajai" lupin seed showed an average daily body mass gain of 2.0 g, those of Group C 2.5 g, and the rats fed diets containing sweet lupin seeds of lower alkaloid content an average body mass gain of 2.3 g/day/animal.

Couch (1926) reported that the various lupin alkaloids were different in toxicity. In toxicity studies on guinea-pigs, d-lupanine proved to be most toxic, followed by sparteine, lupanidine, lupinine and hydroxylupanine. In his review paper on the antinutritive substances of lupins, Mátrai (1977) stressed that the sparteine and lupinidine alkaloids of lupin seeds possessed a pronounced antinutritive effect.

Several authors, including Hackbarth (1961), Pearson and Carr (1977) and Ruiz et al. (1977) drew the conclusion from their experiments that rats were much more resistant to alkaloids than pigs. Thus, depending on the duration of feeding alkaloid-containing diets to pigs, an alkaloid content as low as 0.03–0.1% exerted an adverse effect on their appetite, body mass gain and feed utilization.

In our experiments conducted with rats we have arrived at similar conclusions. Namely, in cases when the alkaloid-containing diets were fed for a short time, the effect of alkaloids could be reduced by supplementing the diet with synthetic amino acids; however, feeding such diets over a prolonged period exerted a definite adverse effect on the studied physiological parameters of the rats.

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EXPERIMENTAL *BACILLUS CEREUS* MASTITIS IN COWS

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After intracisternal inoculation with different doses of *Bacillus cereus* pure cultures, cows developed signs indicative of toxicosis. The symptoms disappeared within 24 h, and an acute parenchymal mastitis developed in the affected udder quarter. The cell count and chloride ion concentration of the milk increased and its consistence markedly changed (mucus, floccules and clots appeared). Subsequently the affected quarter gradually became atrophied and, finally, the milk secretion completely ceased.

Bacteria were detectable in the infected udder quarter in decreasing numbers usually for a week. Repeated attempts to culture bacteria from the blood of the experimental animals consistently failed throughout the experiment. The rabbit skin test and the inoculation of guinea-pigs unequivocally proved that bacterial toxins present both in the milk and in the serum were responsible for the acute symptoms.

The histological examination of biopsy material taken one week post-inoculation revealed vacuolar degeneration of the alveolar epithelium, presence of cell-containing exudate in the lumina and ducts of glands, interstitial oedema, and infiltration by neutrophil granulocytes.

Keywords. *Bacillus cereus*, mastitis, bovine, cow, experimental infection.

During bacteriological examination of raw milk, *Bacillus cereus* has been demonstrated more and more frequently; therefore, the role of this bacterium in the aetiology of mastitis has come to the fore. According to the literature, sporiferous bacteria are infrequent causes of mastitis; however, our knowledge is scarce in this respect. In specialized dairies the adverse hygienic conditions and, mostly, human negligence may render possible for such bacteria to enter the udder. The present studies were undertaken to complete and check our knowledge concerning the udder pathogenicity of *B. cereus*, as well as that concerning the clinical symptoms and milk changes produced by it.

Brown and Scherer (1957) were the first to report the occurrence of *B. cereus* mastitis in a heifer; in that case mastitis was consequent upon udder injury and dilatation of the teat canal with a non-sterile instrument. The affected udder quarters contained a hard, painful swelling, and their skin showed bluish-reddish discolouration. The secretion was yellow or reddish-yellow, serous, and contained fibrin floccules. The applied antibiotic treatment saved the animal's life; however, sequestration occurred in the udder. In a case described by Röhr and Schwarz (1961), *B. cereus* caused a serous, catarrhal-haemorrhagic mastitis, histologically characterized by neutrophil granulocytic infiltration, detachment of epithelial cells, hyperaemia, and interstitial oedema

of the alveoli. Weidlich (1961) diagnosed *B. cereus* mastitis in five cows by bacteriological examination. Histologically he found in the glandular lobules, beside the functioning alveoli, haemorrhagically infiltrated and necrobiotic islets containing numerous bacteria, and thrombosis of blood vessels and lymphatics. The necrotic foci were surrounded by infiltrating lymphocytes and granulocytes. Bacteria were present also in the milk ducts. Gloor (1968) reported focal vacuolar degeneration in the glandular epithelium of the udder, and diffuse infiltration by neutrophil granulocytes in the alveoli. Due to the rapid progression of the disease, this animal was emergency-slaughtered 24 h later. Gloor (1968) inoculated 5 ml of the 24-h broth culture of the *B. cereus* strain isolated from the affected udder into an udder quarter of a cow; 6 h later this was followed by a febrile response (38.5 to 40.6 °C), and the infected udder quarter became swollen, warm and painful. Merck and Burow (1973) reported the occurrence of *B. cereus* mastitis in a freshly-calved cow. With the isolated strain they inoculated a ewe and a cow intracisternally. Both animals developed acute mastitis; the ewe died on the 4th day post-inoculation (PI). In spite of the sulfonamide–aureomycin therapy started 20 h PI, the cow dried off, and the necrotic areas were encapsulated by connective tissue. During gross and histopathological examination of the organs of two cows that had died with symptoms of acute toxaemia, Schiefer et al. (1976) observed in the udder acute inflammation, and haemorrhagic-necrotic foci containing large numbers of Gram-positive bacteria; similar lesions were found in the supramammary lymph nodes. These authors isolated *B. cereus* from the affected udder quarter, found haemoglobin nephrosis in the kidney, and observed hyaline thrombi in the glomerular and lung capillaries. In Bulgaria, Butchvarova and Petrova-Janakieva (1979) proved the involvement of *B. cereus* in the aetiology of mastitis and abortion. Jones and Turnbull (1981) reported 29 cases of *B. cereus* mastitis in cows and one case in a goat. Histologically, they diagnosed focal alveolitis and interstitial oedema in the udder of one of the cows.

Table I

Results of the pre-

Ear tag number		Body temperature °C	Mastitest (CMT)			
			R_F	R_R	L_F	L_R
Experiment I	613	37.9	1+	**1**+	2+	1+
	4526	37.9	2+	2+	**1**+	1+
Experiment II	02	37.9	3+	1+	—	1+
	73	38.2	**2**+	1+	2+	2+
Experiment III	1	37.8	**1**+	3+	2+	1+
	2	38.0	1+	1+	2+	**1**+

The infected quarters are in bold type; R_F = right, front; R_R = right, rear; L_F = left,

Materials and methods

Eight dairy cows were used. A suspension prepared from the pure culture of a *B. cereus* strain isolated from the milk of a cow affected with mastitis was inoculated intracisternally into one udder quarter of two cows each, in three series of experiments. The inoculum contained 10^3 and 10^4; 2×10^3 and 2×10^4; 3×10^7 and 6×10^8; and 2×10^8 and 2×10^7 colony-forming units, respectively, in the four series. Prior to inoculation, the clinical examination of the udder, the Mastitest (CMT) test, the determination of chloride ion concentration (Radelkis OP-261) and cell count (Breed's method) in the milk, as well as the bacteriological examination of milk samples were performed (Table I). Based upon the results of these examinations, the inoculum was injected into the udder quarter best approximating the physiological values; the remaining three quarters served as control. After inoculation, the above-mentioned examinations were repeated first at one-hour intervals, then with decreasing frequency through a period of two weeks; attempts to culture bacteria from the blood of cows were also made. In addition, material obtained by needle biopsy from the infected udder quarter of cows of the first two experiments on PI day 8 was examined histologically. A further series of experiments included the rabbit skin test and inoculation of guinea-pigs to demonstrate toxin effect.

Results

Depending on the infective dose, changes in the general status of cows were observable within 2 to 5 h. The symptoms included listlessness, weakness, anorexia and rise of the body temperature. Between PI hours 5 and 8 the symptoms gradually aggravated, with striking rises of the body temperature, sometimes above 41 °C, and culminated between PI hours 8 and 10. This was followed by a speedy, spontaneous recovery, and by PI hours 22–24 the general

inoculation examinations

Cell count in milk, 10^3/ml				Chloride ion concentration, mmol/l				Bacteriological examination			
R_F	R_R	L_F	L_R	R_F	R_R	L_F	L_R	R_F	R_R	L_F	L_R
2,650	**850**	1,150	1,200	72	**64**	54	44	neg.	**neg.**	neg.	neg.
2,000	2,500	**900**	450	41	23	*32*	31	neg.	neg.	**neg.**	neg.
17,600	600	**1,150**	2,050	50	37	*35*	38	sapr.	neg.	**neg.**	neg.
8,250	7,850	13,400	8,450	*53*	66	62	60	**neg.**	neg.	sapr.	neg.
3,450	900	2,050	1,450	**32**	27	32	34	**neg.**	neg.	neg.	neg.
1,850	16,500	20,000	**2,650**	27	40	32	**27**	neg.	sapr.	sapr.	**neg.**

front; L_R = left, rear quarter

Table II

Cell counts in the milk of two cows following experimental infection (10/ml)
Infected quarters: 613/R_R with 10^3 CFU and 4526/L_F with 10^4 CFU

Time post-inoculation (h) Udder quarter		0	2	5	8	10	24	29
R_F*	613	2,650	11,200	11,250	13,100	4,250	650	700
R_R	613	850	7,500	7,950	27,600	30,300	24,000	19,950
L_F	613	1,150	3,850	4,700	4,950	6,450	700	1,800
L_R	613	1,200	8,900	1,500	2,250	1,000	550	250
R_F	4526	2,000	4,100	2,250	3,150	950	700	500
R_R	4526	2,500	16,400	8,300	13,300	5,200	1,000	500
L_F	4526	900	3,250	31,500	50,000	54,900	12,450	11,500
L_R	4526	450	2,100	2,400	9,600	600	450	550

* For explanation, see Table I

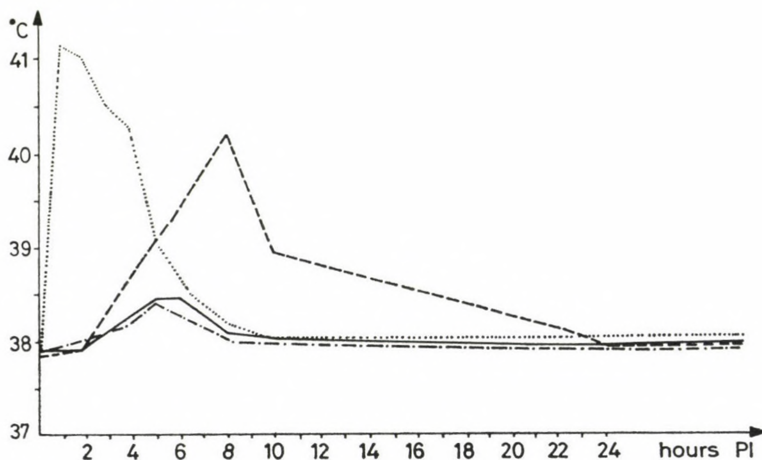

Fig. 1. Body temperature after infection (infective dose: ——— 10^3 CFU; -·-·-·-·- 2×10^3 CFU; --------- 10^4 CFU; ······ 3×10^7 (CFU)

Table III

Results of the post-inoculation examinations

Ear tag number		Body temp. °C	Mastitest (CMT)			
			+R_F	R_R	L_F	L_R
Experiment I	613	38.0	2+	**3+**	1+	1+
	4526	38.8	1+	2+	**3+**	1+
Experiment II	02	38.1	3+	1+	**3+**	3+
	73	39.2	**3+**	2+	3+	3+
Experiment III	1	39.2	**3+**	3+	2+	1+
	2	39.2	1+	3+	2+	**3+**

Infected udder quarters are in bold type

status and body temperature returned to normal (Fig. 1). Thus, no medicinal treatment was needed to save the animals' life. Parallel to the appearance of clinical symptoms, an acute parenchymal mastitis accompanied by pronounced oedema, reddening and pain, developed in the infected udder quarters. The cows hardly tolerated even hand milking. From the infected quarters a yellowish-white, turbid, viscous secretion, containing lumps of mucus and floccules, could be milked as early as PI hour 5. Later on the milk proteins and the butterfat precipitated, rendering the udder quarters hard to milk. By that time the colour of the secretion had changed to yellowish-greenish. After PI hours 22–24 udder oedema ceased to exist, skin hyperaemia was less expressed, and milking became undisturbed; however, the colour and consistence of the milk remained abnormal, and its quantity decreased.

Depending on the infective dose, the cell count of the milk reached 50 to 80 million per ml by PI hour 8. By PI hours 8 to 10, chloride ion con-

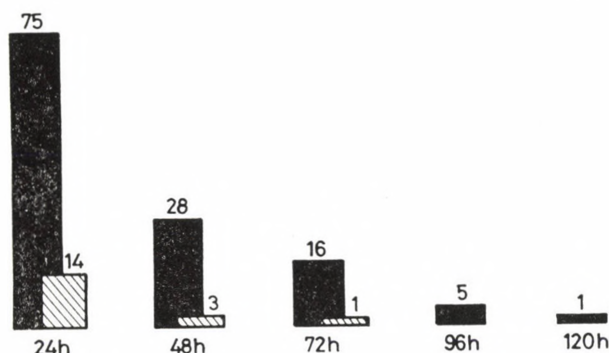

Fig. 2. Number of *B. cereus* colonies cultured from the samples, depending on the infective dose (Experiment 2; striated columns: 2×10^3 CFU; black columns: 2×10^4 CFU)

(means of values measured in the first 24 h)

Cell count in milk (10^3/ml)				Chloride ion (mmol/l)			
R_F	R_R	L_F	L_R	R_F	R_R	L_F	L_R
7,133	**20,800**	3,808	2,450	55.6	**68.6**	47.5	43.8
2,408	7,950	**29,175**	2,608	58.3	51.8	* not measurable	44.2
20,937	2,725	**11,775**	29,587	73.5	45.9	**86.1**	96.1
57,437	9,537	16,525	13,950	*	71.3	79.4	68.5
25,094	35,187	7,131	2,406	**98.4**	77.3	53.1	57.3
2,993	16,087	9,376	**15,137**	15.6	80.6	78.3	**68.8**

* Not measurable by the method applied; for explanation, see Table I

centration reached a value of 100 to 120 mmol/l; then, in many cases, it was immeasurable because the mucous, flocculent milk could not be sucked up into the capillary of the Radelkis OP-50 chloride ion meter (Tables II and III). Subsequently, both the cell count and, later on, the chloride ion concentration decreased; however, both persisted at rather high levels through a period of several days, depending on the germ count of the inoculum. The cell count persisted at 20 to 25 million per ml, and the chloride ion concentration at 70 to 100 mmol/l. Elimination of *B. cereus* bacteria from the udder took 72 to 120 hours (Fig. 2). The quality of the abnormally altered secretion did not change during the subsequent 3 to 6 weeks. During this period the affected udder quarter gradually became atrophied and dried off.

The blood samples taken repeatedly after infection were bacteriologically negative, suggesting the involvement of a possible toxin effect. A further experiment was conducted to verify this suggestion. In this experiment milk and blood samples were taken 8 h after the onset of clinical symptoms. After centrifuging the samples, the milk proteins were removed by precipitation and after subsequent filtration, the pH of the filtrate was adjusted to 6.8. Blood serum and milk whey from a healthy cow were used as control. The test materials were rubbed into the shaved back skin of two rabbits at four sites. The blood serum and milk whey of the cow inoculated with 2×10^8 CFU were rubbed into the skin on the left side, whereas the control materials on the right side. The other rabbit was treated with the blood serum and milk whey of the cow inoculated with 2×10^7 germs. While on the "control" side only a slight reddening of the skin occurred 24 h later, by that time the test materials produced a painful red swelling 4 to 5 cm in diameter. The biological tests included inoculation of guinea-pigs: each guinea-pig received 0.1 ml blood serum intramuscularly. The guinea-pig inoculated with the blood serum of the cow that had received the larger inoculum became febrile within 24 h. The body temperature of the guinea-pig treated with the serum of the cow given the smaller inoculum rose, but after 24 h the animal's appetite and body temperature returned to normal. The general status of the guinea-pig inoculated with the control serum remained unchanged.

In the first and second series of experiments, histological examination of samples taken from the infected udder quarters by needle biopsy on PI day 8 revealed an exudate containing neutrophilic granulocytes in the dilated alveoli and ducts, vacuolation in the glandular epithelium, as well as oedema and infiltration by neutrophilic granulocytes in the interstices (Figs 3 and 4).

Discussion

Experimental infection with *B. cereus* resulted in a gradual deterioration of the cows' general status from PI hours 2–5 onwards. The symptoms culminated between PI hours 8 and 10. The animals became listless, weak, showed anorexia and, in some cases, their body temperature rose as high as 41 °C. Subsequently the symptoms gradually subsided, and by PI hours 22 to 24 the general status returned to normal. On the other hand, from PI hour 5 an acute parenchymal mastitis developed, accompanied by a striking change of the secreted milk. Milk changes persisted for 3 to 6 weeks, during which period the affected quarter became atrophied and dried off. The present observations support those of Gloor (1968) and Merck and Burow (1973) on the development of acute mastitis.

Schiefer et al. (1976) reported the development of mastitis and acute toxicosis following *B. cereus* infection. The presence of toxicosis has been confirmed by the rabbit skin test and inoculation of guinea-pigs also in the present experiments; however, we have found that toxaemia lasted only 24 h. In *B. cereus* mastitis, histological examinations were performed first by Röhr and Schwarz (1961), then by Gloor (1968), Schiefer et al. (1976) and, finally, by Jones and Turnbull (1981). These authors reported a focal infiltration of the alveoli by neutrophilic granulocytes, vacuolation of epithelial cells, interstitial oedema, and development of necrotic foci. The examination of the biopsy samples taken by us yielded similar results, with the only difference that we failed to find necrosis. This fact might be attributed to a possible low hit probability of the needle biopsy technique. Our experiments furnish new data on the cell count and chloride ion concentration of the milk secreted in *B. cereus* mastitis.

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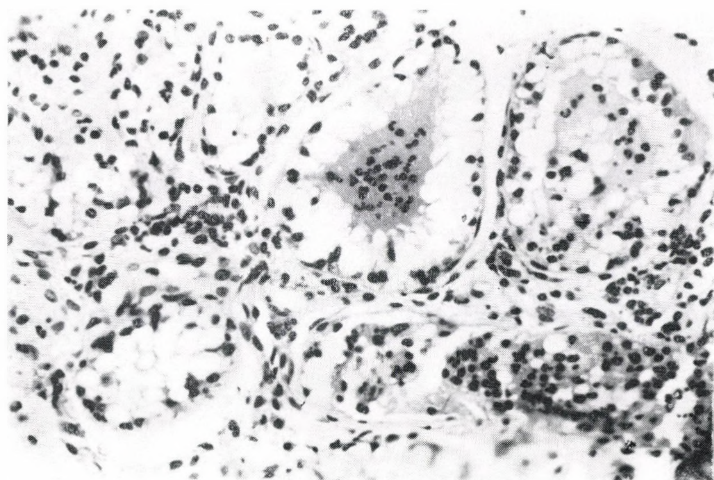

Fig. 3. Vacuolar degeneration in the alveolar epithelium of the lactiferous gland, serous exudate and smaller and larger groups of neutrophilic granulocytes in the lumina of glands and in the dilated efferent duct. Note infiltration by neutrophilic granulocytes in the interstices (biopsy material). Haematoxylin–eosin, appr. ×250

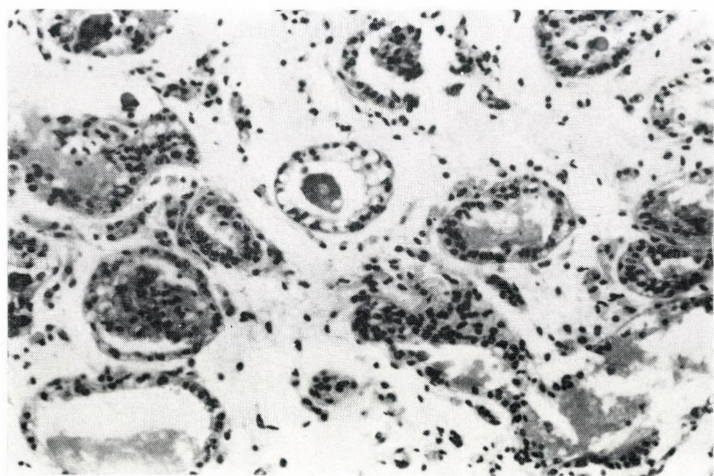

Fig. 4. Serous-cellular exudate in the dilated alveoli of the udder; interstitial oedema (biopsy material). Haematoxylin-eosin, appr. ×80

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INFECTION EXPERIMENTS WITH *MYCOBACTERIUM PARATUBERCULOSIS* STRAINS

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The pathogenic and immunogenic properties of *Mycobacterium paratuberculosis* strains isolated from cattle were studied in rabbits, day-old chickens and turkeys, and compared with the characteristics of *M. paratuberculosis* and *M. avium* reference strains.

Rabbits inoculated intravenously with suspensions (extinction values: 0.07 and 0.55) of the *M. paratuberculosis* reference strain no. 5889 developed histopathological lesions indicative of mycobacterial action, whereas in rabbits given smaller inocula (extinction value: 0.01) of the same strains no such lesions were seen. Of the *M. paratuberculosis* strains isolated from cattle, three produced histopathological lesions after either intravenous or subcutaneous administration, while 2 strains only when injected intravenously. Six of the 19 rabbits inoculated developed complement-fixing (CF) antibodies in their sera. The agar gel precipitation test (AGPT) was consistently negative, while the lymphocyte-stimulation test (LST) positive in all the animals tested.

In chickens and turkeys the development of gross and histopathological lesions depended on the size of the inoculum. No antibodies were demonstrable in the serum with the methods applied; however, the LST was positive.

The histopathological lesions and serological responses occurring in rabbits inoculated intravenously with the *M. avium* strains used for comparison were essentially similar, depending on the size of the inoculum and on the route of applications, and were identical with the lesions produced by *M. paratuberculosis*.

Keywords. *Mycobacterium paratuberculosis*, *M. avium*, cattle, experimental infection, pathogenicity, immunogenicity, histopathology, rabbit, chicken, turkey.

In recent years new data have been obtained on the pathological role of *Mycobacterium paratuberculosis*. However, the data on the virulence of strains isolated from different species are inconsistent, frequently even contradictory. According to Lominski et al. (1956), the pathological lesions caused by intravenously administered *M. paratuberculosis* differ from those following oral infection with the same organism. In their comparative studies on *M. paratuberculosis*, *M. avium* and *M. intracellulare* strains by different methods (mortality, gross pathology, and allergy tests in calves, rabbits, chickens, guinea-pigs and mice), Collins et al. (1983) drew the conclusion that the applied methods were unsuitable for characterizing the virulence of the isolates.

In the present investigations the properties of *M. paratuberculosis* strains isolated from cattle were studied in infection experiments conducted with rabbits, chickens and turkeys, in comparison to those of standard *M. paratuberculosis* and *M. avium* strains. We attempted to correlate the produced

pathological lesions with the route of infection and the infective dose. Furthermore, the humoral and cellular immune responses of the experimental animals were studied.

Materials and methods

Infection experiments in rabbits

Of the reference strains, different densities (extinction values: 0.55; 0.07; 0.01; Specol Zeiss Jena 450 nm) of the *M. paratuberculosis* reference strain designated 5889 Prague, and of the *M. avium* reference strain designated 16909-3380 ATCC (extinction values: 1.9; 0.33; 0.04) were used. With 1 ml live suspension of each density grade of *M. paratuberculosis* two rabbits, while with that of *M. avium* one rabbit (body mass: 3000 to 4000 g) was inoculated intravenously. The rabbits were bled on post-inoculation (PI) day 15 and 14, respectively. The blood samples were examined by the lymphocyte-stimulation test (LST), the complement fixation test (CFT), and the agar gel precipitation test (AGPT). After gross pathological examination, histological sections were prepared from the spleen, liver, and ileum. The sections were stained with haematoxylin and eosin, and with the Ziehl–Neelsen (ZN) stain.

One rabbit was inoculated intravenously, another subcutaneously, each with 1 ml of live bacterial suspensions (extinction values ranging between 0.30 and 0.41 for the different strains) of 5 *M. paratuberculosis* strains (designated 599/81 G, 601/81 Sz, 663/81 P, 7/82 Sz, and 104/82 O) isolated in Hungary and identified according to Thorel and Valette (1976). The rabbits were kept under observation for a month and subsequently killed by bleeding. Their organ and blood samples were examined as described above.

Infection of day-old chickens and turkeys

Seven day-old chicks were inoculated subcutaneously with 0.5-ml suspensions of *M. paratuberculosis* strain 5889 and *M. paratuberculosis* Weybridge, 7/82 Sz, 599/81 G, 601/81 Sz, and 663/81 P live strains (extinction values: 0.87; 0.46; 0.51; 0.39; and 0.32). Two 3 weeks old turkeys were infected per os on three occasions (on two successive days and one week thereafter) with the *M. avium* strain designated 16909-3380 ATCC. For per os infection a homogeneous mash was prepared from a 4-week-old Sauton culture and the flour-fine mixed-feed grist. The birds were given only this feed and drinking water, both ad libitum. Furthermore, one 3 weeks old turkey each was inoculated subcutaneously with a 1-ml live bacterial suspension (extinction value: 0.14) prepared from strain 599/81 G, 7/82 Sz, and the *M. paratuberculosis* reference strain designated 5889 Prague, respectively. The samples were processed as described under the heading "Infection experiments in rabbits".

Antigens

The complement-fixing antigen was prepared according to the method of Gorrie (1959) from the *M. paratuberculosis* Bergey 1923 strain derived from Prague.

The gel-precipitation antigen was prepared from 4-week-old Sauton broth cultures of the same *M. paratuberculosis* strain, sterilized by filtration through Seitz filter. The cultures were condensed to contain 6.93 mg/ml dry matter (Körmendy et al., 1984).

For the LST, 1 mg/ml PPD tuberculin (50,000 IU/ml) prepared from the AN_5 strain of *M. bovis*, and 0.5 mg/ml (25,000 IU/ml) purified tuberculoprotein prepared from the D4 strain of *M. avium* were used as antigens.

Complement fixation (CF) test

In the main experiment, 1% excess of complement was added to the lowest complement dilution causing full haemolysis. Sera to be tested, diluted 1 : 10, were inactivated at 58 °C for 30 min. The CF test was carried out for 2×20 min in a waterbath of 37 °C. The tests displaying no haemolysis in the dilution of 1 : 10 were considered positive.

Gel precipitation test (AGPT)

The test was performed in 0.8% agarose prepared in 0.15 M Sorensen's phosphate buffer of pH 7.0. In the double radial gel diffusion test the volume of the reservoirs was 0.075 cm^3. The reactions were read daily on 5 successive days.

Lymphocyte-stimulation test (LST)

Leucocytes separated from the blood samples with Ficoll-Paque were resuspended in Hanks' solution containing 10% fetal calf serum and antibiotics, up to a cell density of 10^6. One and a half ml volumes of the cell suspension were measured into Leighton tubes to each of which 200 μg antigen solution was added. Into the control tubes 0.1 ml PBS was measured. In the 57th h of incubation at 37 °C, 10 μCi tritiated thymidine was added to each tube containing antigen-stimulated cell cultures. Sixteen h later the plates were fixed in methanol and washed with PBS. Subsequently the plates were immersed in Ilford-Nuclear K2 emulsion, dried, and the dry plates were kept in dark-room at 4 °C for 6 days. The plates were then developed in 49-ORWO solution, fixed, stained according to May–Grünwald, and examined in light microscope.

Histopathological studies

Frozen sections prepared from spleen, liver and intestine samples fixed in 10% neutral formalin were stained with haematoxylin and eosin. The Ziehl–Neelsen stain was used for demonstrating mycobacteria in tissue sections.

Results

The results of the experiments conducted in rabbits are shown in Tables I and II.

In rabbits inoculated with the *M. paratuberculosis* reference strain a swelling of the spleen appeared on PI day 14; by histological examination both the spleen and the liver samples showed tissue changes characteristic of mycobacterial infection. Mycobacteria were demonstrated in the ileum of two animals.

Of the rabbits infected with *M. avium*, rabbits nos 7 and 8 developed essentially similar lesions. However, in rabbit no. 9, which had received the smallest inoculum, the histopathological lesions were restricted to the spleen.

Infection with the *M. paratuberculosis* strains isolated from cattle failed to produce gross lesions in rabbits bled on PI day 30. By histological examination, however, most of the rabbits showed lymphoid cell proliferation in the liver and spleen, with the presence of Langhans-type giant cells, and acid-fast bacteria in a nest-like arrangement (Table II).

The AGPT was negative for all animals, while the CFT was positive for three animals in each group (Tables I and II). On the other hand, the LST gave positive results with all samples tested, except for rabbit no. 9 (Table I).

The results of the experiments conducted in chickens are shown in Table III.

In the spleen of birds infected with suspensions having extinction values between 0.46 and 0.87, histopathological examination revealed hypertrophy of Malpighian bodies, lymphoid cell proliferation and, with the exception of chicken no. 4, masses of acid-fast bacteria showing nest-like arrangement. In the liver of chicks nos 1, 2 and 3, in addition to fatty degeneration, there were clumps of mononuclear lymphoid cells around the central veins and conglomerates of acid-fast bacteria. In these chicks, except for chicks nos 1 and 4, numerous mononuclear cells were observed among the connective tissue elements of the ileal mucosa, and the solitary lymphoid follicles of the submucosa became enlarged because of the proliferation of lymphoid cells. In these places acid-fast bacteria were seen either singly or in nest-like arrangement.

No gross pathological lesions were found in birds nos 6 and 7, i.e. the birds infected with a more diluted inoculum. Histopathology revealed proliferation of lymphoid tissues in these organs, without the presence of acid-fast bacteria.

The results of the experiments conducted in turkeys are shown in Table IV.

In the 10th week after *M. avium* infection, although no gross lesions were present, histopathological examination revealed proliferation of lymphoreticular tissue in the Malpighian bodies of the spleen and a perivascular

Table I

Results obtained for rabbits inoculated with reference strains

Serial number of rabbits	Mycobacterium strain	Bacterium density (extinction)	Time of inoculation and killing	Gross pathology L = liver S = spleen I = intestine	Histopathology		Blood tests		LST	
					H–E.	ZN	CFT	AGPT	*M. avium* PPD	AN_5 PPD
Rabbit 1	*M. paratuber-culosis* 5889 1 ml, iv.	0.55	24. 05. 82 died 07. 06	L: — S: swollen I: —	L: ly.g. S: ly.g. I: —	+ + —	not tested			
Rabbit 2	*M. paratuber-culosis* 5889 1 ml, iv.	0.55	24. 05. 82 08. 06. 82	L: — S: swollen I: —	L: ly.g. S: ly.g. I: ly.g.	+ + +	+ —	—	12	6
Rabbit 3	*M. paratuber-culosis* 5889 1 ml, iv.	0.07	24. 05. 82 08. 06. 82	L: — S: swollen I: —	L: ly.g. S: ly.g. I: ly.g.	+ + +	—	—	10	4
Rabbit 4	*M. paratuber-culosis* 5889 1 ml, iv.	0.07	24. 05. 82 08. 06. 82	L: — S: swollen I: —	L: ly.g. S: ly.g. I: —	+ + —	+	—	19	7
Rabbit 5	*M. paratuber-culosis* 5889 1 ml, iv.	0.01	24. 05. 82 08. 06. 82	L: — S: swollen I: —	L: ly.g. S: ly.g. I: —	— — —	—	—	21	6
Rabbit 6	*M. paratuber-culosis* 5889 1 ml, iv.	0.01	24. 05. 82 08. 06. 82	L: — S: swollen I: —	L: ly.g. S: ly.g. I: —	— — —	+ —	—	6	11
Rabbit 7	*M. avium* 16909 13380 1 ml, iv.	1.9	24. 05. 82 07. 06. 82	L: — S: swollen I: —	L: — S: ly.g. I: ly.g.	+ + +		—	29	
Rabbit 8	*M. avium* 16909 13380 1 ml, iv.	0.33	24. 05. 82 07. 06. 82	L: — S: swollen I: —	L: ly.g. S: ly.g. I: —	+ + —	+ anticlp.	—	25	17
Rabbit 9	*M. avium* 16909 13380 1 ml, iv.	0.04	24. 05. 82 07. 06. 82	L: — S: swollen I: —	L: — S: ly.g. I: —	— — —	anticlp.	—	2	2

anticlp. = anticomplementary; ZN = acid-fast bacteria staining with Ziehl–Neelsen's stain; H–E. = haematoxylin–eosin; ly.g. = lymphoid granuloma

Table II

Results obtained for rabbits inoculated with strains isolated from cattle

Rabbits	Mycobacterium strain	Bacterium density (extinction)	Time of inoculation and killing	Gross pathology L = liver S = spleen I = intestine	Histopathology		Blood tests		LST
					H–E.	ZN	CFT	AGPT	*M. avium* PPD
Rabbit 1	*M. paratuberculosis* 599/81 G 1 ml, iv.	0.30	29. 06. 82– died 26. 07. 82	L: — S: swollen I: —	L: ly.g. S: ly.g. I: —	+ +		not tested	
Rabbit 2	*M. paratuberculosis* 599/81 G 1 ml, sc.	0.30	29. 06–29. 07	L: — S: — I: —	L: ly.g. S: ly.g. I: —	— — —	+	—	16
Rabbit 1	*M. paratuberculosis* 601/81 Sz 1 ml, iv.	0.30	29. 06–29. 07	L: — S: — I: —	L: ly.g. S:— ly.g. I: —	— —	—	—	20
Rabbit 2	*M. paratuberculosis* 601/81 Sz 1 ml, sc.	0.30	29. 06–29. 07	L: — S: — I: —	L: ly.g. S: ly.g. I: —	—	+	—	14
Rabbit 1	*M. paratuberculosis* 663/81 P 1 ml, iv.	0.31	29. 06–29. 07	L: — S: — I: —	L: — S: — I: —	— — —	—	—	12
Rabbit 2	*M. paratuberculosis* 663/81 P 1 ml, sc.	0.31	29. 06–29. 07	L: — S: — I: —	L: — S: — I: —	— — —	—	—	12
Rabbit 1	*M. paratuberculosis* 7/82 Sz 1 ml, iv.	0.41	29. 06–29. 07	L: — S: — I: —	L: ly.g. S: ly.g. I: —	— — —	+	—	28
Rabbit 2	*M. paratuberculosis* 7/82 Sz 1 ml, sc.	0.41	29. 06–29. 07	L: — S: — I: —	L: — S: ly.g. I: —	— —	—	—	not tested
Rabbit 1	*M. paratuberculosis* 104/82 O 1 ml, iv.	0.35	29. 06–29. 07	L: — S: — I: —	L: ly.g. S: ly.g. I: —	— — —	—	—	9
Rabbit 2	*M. paratuberculosis* 104/82 O 1 ml, sc.	0.35	29. 06–29. 07	L: — S: — I: —	L: — S: — I: —	— —	—	—	not tested

H–E. = haematoxylin–eosin; ZN = Ziehl–Neelsen stain; ly.g. = lymphoid granuloma

Table III

Results of infection experiments in chickens

Serial number of chickens	Mycobacterium strain and route of infection	Bacterium density (extinction)	Time of inoculation and killing	Gross pathology L = liver S = spleen I = intestine	Histopathology		Blood tests		LST at killing
					H–E.	ZN	CFT	AGPT	*M. avium* PPD
Chicken 1	*M. paratuberculosis* 5889 0.5 ml, sc.	0.87	01. 04. 82–04.06	L: foci S: swollen I: —	L: ly.g. S: ly.g. I: —	+ +	—	—	not tested
Chicken 2	*M. paratuberculosis* 5889 0.5 ml, sc.	0.87	01. 04–04. 06	L: foci S: swollen I: —	L: ly.g. S: ly.g. I: ly.g.	+ + +	—	—	not tested
Chicken 3	*M. paratuberculosis* W. 0.5 ml, sc.	0.46	17. 05–28. 06	L: — S: swollen I: —	L: ly.g. S: ly.g. I: ly.g.	+ + +	—	—	9
Chicken 4	*M. paratuberculosis* 7/82 Sz 0.5 ml, sc.	0.51	17. 05–11. 06	L: — S: swollen I: —	L: ly.g. S: ly.g. I: —	— + —	—	—	32
Chicken 5	*M. paratuberculosis* 599/81 G 0.5 ml, sc.	0.50	17. 05–11. 06	L: — S: swollen I: —	L: ly.g. S: ly.g. I: —	— + —	—	—	not tested
Chicken 6	*M. paratuberculosis* 601/81 Sz 0.5 ml, sc.	0.39	17. 05–28. 06	L: — S: — I: —	L: ly.g. S: ly.g. I: ly.g.	— — —	—	—	20
Chicken 7	*M. paratuberculosis* 663/81 P 0.5 ml, sc.	0.32	17. 05–28. 06	L: — S: — I: —	L: ly.g. S: ly.g. I: ly.g.	— — —	—	—	16

H–E. = haematoxylin–eosin; ZN = Ziehl–Neelsen stain; ly.g. = lymphoid granuloma

Table IV

Results of infection experiments in turkeys

Serial number of turkeys	Bacterium strain and route of infection	Bacterium density (extinction)	Time of inoculation and killing	Gross pathology L = liver S = spleen	Histopathology		Blood tests		LST
					H–E.	ZN	CFT	AGPT	*M. avium* PPD
Turkey 1	*M. avium* 16909-3380 per os	—	14. 07. 82– 15. 07–21. 07– 28. 09	L: —	L: ly.g.	—	—	—	12
Turkey 2	*M. avium* 16909–3380 per os	—	14. 07–15. 07– 21. 07–28. 09	L: — S: —	L: ly.g. S: ly.g.	— —	—	—	8
Turkey 3	*M. paratuberculosis* 599/81 G 1 ml, sc.	0.14	15. 07–28. 09	L: small foci S: swollen	L: ly.g. S: ly.g.	— —	—	—	16
Turkey 4	*M. paratuberculosis* 7/82 Sz 1 ml, sc.	0.14	15. 07–28. 09	L: — S: —	L: — S: —	—	—	—	6
Turkey 5	*M. paratuberculosis* 5889 1 ml, sc.	0.14	15. 07–28. 09	L: small foci S: swollen	L: ly.g. S: ly.g.	— —	—	—	not tested

H–E. = haematoxylin–eosin; ZN = Ziehl–Neelsen stain: ly.g. = lymphoid granuloma

proliferation of lymphoid cells in the liver. However, no acid-fast bacteria were observed.

Similar histopathological changes were found in the liver and spleen of turkeys infected subcutaneously with *M. paratuberculosis* strains 599/81 G, 7/82 Sz and the *M. paratuberculosis* reference strain 5889 Prague.

Discussion

During investigations into the pathogenicity of *M. paratuberculosis* strains in different laboratory animals it has already been observed that the gross and histopathological changes produced by them depend on the route of infection (Lominski et al., 1956). Thus, no conclusions as to the virulence of strains can be drawn from these changes.

In the present infection experiments we studied the pathological lesions produced in rabbits, chickens and turkeys as a function of the size of inoculum. Parallel to this, we assayed the humoral and cellular immune response developing in the experimental animals.

In rabbits infected intravenously with suspensions of the *M. paratuberculosis* reference strain no. 5889 (extinction values: 0.55 and 0.07; Table I) a swelling of the spleen appeared on day 14. Histologically, lesions characteristic of mycobacterial infection were observed in both the spleen and the liver. In rabbits given a dilute inoculum (extinction value: 0.01) of the same strain the spleen was swollen, but histologically only a proliferation of lymphoid cells around the central veins of the liver and an enlargement of the Malpighian bodies of the spleen could be observed. Neither Langhans-type giant cells nor acid-fast bacteria were seen in these lesions.

Of the rabbits inoculated with different doses of the *M. avium* strain, the gross and histopathological findings in rabbit no. 7 (Table I) were consistent with those in rabbits nos 1, 2, 3 and 4. The gross and histopathological picture of the spleen and liver of rabbit no. 8 was similar to that in rabbits nos 5 and 6. In rabbit no. 9 infected with a suspension having an extinction value of 0.04 only the spleen sample showed histopathological changes suggestive of mycobacterial infection.

The *M. paratuberculosis* strains isolated from cattle failed to cause gross lesions in rabbits inoculated intravenously or subcutaneously and autopsied on PI day 30, with the exception of one rabbit (Table II). The rabbit inoculated intravenously with strain 599/81 G died on day 27 PI. Upon the gross and histopathological examination of this rabbit a hyperaemic swelling of the spleen, lymphoid cell foci around the central veins of the liver, enlargement of the Malpighian bodies and lymphoid cell proliferation in the spleen, a few Langhans-type giant cells and numerous acid-fast bacteria, dispersed or in

nest-like arrangement, were found. In the rabbits inoculated with strain 601/81 Sz intravenously or subcutaneously, the above-listed histopathological changes developed in the liver and spleen, in animals injected with strain 7/82 intravenously also in the liver and spleen, while in those given the latter strain subcutaneously only in the spleen.

Strain 104/82 O produced lesions only when administered intravenously, whereas strain 663/81 P failed to cause lesions either intravenously or subcutaneously.

With the exception of one rabbit, no acid-fast bacteria could be demonstrated in the spleen, liver and ileal portion of rabbits shown in Table II, irrespective of whether tissue reactions indicative of mycobacteria were present or absent.

Of the 19 rabbits infected with inocula of different sizes of *M. paratuberculosis* strains, 6 animals developed complement-fixing antibodies (Tables I and II). The AGPT was consistently negative.

The rate of in vitro blastogenesis determined by the LST showed that all the examined animals infected with the *M. paratuberculosis* reference strain were in a sensitized state. Antigenic stimulation with *M. avium* resulted in a higher rate of blastogenesis than the antigen of *M. bovis* AN_5 strain (Table I). Of the rabbits inoculated with *M. paratuberculosis* strains isolated from cattle, 7 were subjected on day 30 to LST in which *M. avium* PPD was used. The number of lymphoblast cells ranged between 9 and 28, and in this experimental design the test indicated a post-inoculation immune response. Although infection with strain 663/81 P failed to produce a tissue response in the rabbits, the LST was positive. The strains could not be differentiated by using the LST.

In day-old chickens and turkeys the produced gross and histopathological lesions depended on the size of the inoculum (Tables III and IV). No antibodies were demonstrable in the sera of experimental birds with the serological methods applied; however, the rate of blastogenesis assessed by the LST indicated a state of sensitization. Summing up the results of these experiments, it appears that *M. paratuberculosis* strains may cause in the liver, spleen and ileum a focal tissue reaction, most frequently appearing as a proliferation of lymphoid cells and occasionally containing Langhans-type giant cells. These lesions resemble those occurring in tuberculosis; however, they are free from necrosis and calcification. The produced histopathological lesions depend on the size of inoculum and the route of infection.

At the conclusion of the experiments, the CFT indicated infection only in a rather small proportion of cases: of the 16 and 3 rabbits infected with *M. paratuberculosis* and *M. avium*, respectively, 5 gave a positive reaction, two gave a doubtful reaction, which may be regarded as group reactions due to antigenic relatedness (Table I).

The LST is sensitive and suitable for assessing the state of sensitization developing after infection. With purified antigens, this test could be used even for differentiation between strains causing infection.

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BIOCHEMICAL AND SEROLOGICAL CHARACTERISTICS OF *CAMPYLOBACTER JEJUNI* STRAINS ISOLATED FROM CHICKENS

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Biochemical and serological characteristics of 62 campylobacter strains isolated from cases of chicken campylobacter (vibrionic) hepatitis, from the caecum of clinically healthy hens, and from the surface of slaughtered chickens are described. The tube agglutination test was used for the serological classification with heated (10 min at 100 °C) antigens. Typing sera were prepared in rabbits against several *Campylobacter jejuni* isolates.

In three flocks affected with campylobacter hepatitis egg production dropped by 8.7, 14.1 and 16.4%, respectively. About 5% of the birds suffered from diarrhoea but the rate of mortality did not exceed 2.3% on the average of the three flocks.

Sixty strains were identified as *C. jejuni*, one as *C. coli*, and one belonged to the nalidixic-acid-resistant thermophilic Campylobacter group. Most of the *C. jejuni* strains showed typical growth and biochemical characteristics, but some did not grow in the presence of glycine, triphenyltetrazolium chloride (TTC) or sodium selenite.

According to the heat-stable antigens, 34 out of 41 *C. jejuni* strains could be assignod into one of six serogroups.

Keywords. Chicken, campylobacter, hepatitis, *Campylobacter jejuni*, biochemical and serological characteristics.

Avian campylobacter (vibrionic) hepatitis, a disease which appears most often in laying hens, causes significant drop in egg production and low mortality rates. Since its first recognition (Hofstad et al., 1958; Peckham, 1958; Winterfield et al., 1958) the disease has been described in several countries (Bisping et al., 1963; Köbl, 1964; Bertschinger, 1965; Palya et al., 1971). From the liver, gall-bladder, heart blood and intestine of the succumbed chickens microaerophilic vibrios were isolated, which in their morphological, cultural and biochemical characteristics were very similar (Peckham, 1958) to King's (1957) related vibrio isolated from children with diarrhoea. Microaerophilic vibrios causing hepatitis in chickens were resistant to bacitracin, polymyxin-B (Winterfield et al., 1958) and, like *Vibrio fetus* strains cultured from cattle (Plastridge and Koths, 1961), also to novobiocin. These three antibiotics added to thioglycollate medium enabled the selective isolation of microaerophilic vibrios from chickens (Winkenwerder and Bisping, 1964; Winkenwerder and Maciak, 1964).

Recently microaerophilic vibrios are assigned to the genus *Campylobacter* (Véron and Chatelain, 1973; Smibert, 1978) and the strains isolated from avian vibrionic hepatitis are now considered to be *C. jejuni* and *C. coli*. During the past ten years it has also been learned that the distribution of *C. jejuni*

is much wider than it was thought (Smibert, 1978; Skirrow and Benjamin, 1980; Skirrow, 1982; Garcia et al., 1983). They may cause not only avian campylobacter hepatitis but also abortion and infertility in sheep (Berg et al., 1971; Smibert, 1978; Garcia et al., 1983), enteritis in dogs and cats (Skirrow, 1981) and very often enteritis with diarrhoea in man (Skirrow and Benjamin, 1980; Skirrow, 1982; Blaser et al., 1983). Their significance in the aetiology of human enteritis has become evident since the introduction of antibiotic-containing media for their selective isolation from human faeces (Butzler et al., 1973; Skirrow, 1977; Lauwers et al., 1978). *C. jejuni* is a normal inhabitant of the intestines of most animals, including several species of birds (Smibert, 1978; Skirrow and Benjamin, 1980; Skirrow, 1982; Blaser et al., 1983; Garcia et al., 1983). *C. jejuni*-carrying animals and their food products, among them very often raw chicken meat, may transmit infection to the human consumer, therefore the disease seems to be a zoonosis (Skirrow, 1982; Blaser et al., 1983).

The purpose of this study was to examine biochemical and serological characteristics of campylobacter strains isolated from laying hens succumbed to campylobacter hepatitis and from the meat of slaughtered chickens.

Materials and methods

Source of strains. Sixty-two campylobacter strains were examined. Out of them 15 were isolated from the liver, gall-bladder or small intestine of laying hens succumbed to campylobacter hepatitis in flocks of three farms. Further 7 strains were isolated from the caecum of clinically healthy hens, while 40 were recovered from cotton swabs taken from the surface of slaughtered chickens at the end of the dressing line.

Cultural methods, biochemical and antibiotic sensitivity tests. All strains were isolated on selective blood agar containing vancomycin, polymyxin-B and trimethoprim (Skirrow, 1977) and growth-promoting supplement (George et al., 1978). The cultures were incubated under microaerophilic conditions (6% O_2, 10% CO_2 and 84% N_2) at 37 °C. Growth and biochemical characteristics, except for H_2S production, triphenyltetrazolium chloride (TTC) and sodium selenite reduction, were examined in thiol medium (Difco) according to standard criteria (Véron and Chatelain, 1973; Smibert, 1978). For H_2S production brucella broth (Difco) with 0.1% agar and with 0.02% cysteine, and TSI (*t*riple *s*ugar *i*ron) agar (Difco) were used. Growth in the presence and reduction of 0.1% TTC and of sodium selenite were tested on blood agar. Hippurate hydrolysis was examined according to the rapid method of Hébert et al. (1982). All semisolid media were incubated at normal atmospheric conditions. Antibiotic sensitivity of 10 selected strains was examined on blood agar with discs containing antibiotics (Resistest Human).

Serological examinations. Forty-one strains were examined by the tube agglutination test. Antisera were prepared in rabbits against several *C. jejuni* isolates; fresh cultures were washed off with saline containing 0.15% formalin. Each rabbit received a series of intravenous inoculations at intervals of 3–4 days. As antigen for the tube agglutination test suspensions of campylobacter strains washed off with saline were heated for 10 min at 100 °C. The test was read after an incubation at 37 °C for 24 h.

Results

Egg production decreased by 8.7, 14.1 and 16.4%, respectively, during the 3-week existence of clinical signs of campylobacter hepatitis in the 3 flocks examined. About 5% of the birds were affected with diarrhoea, but the mortality did not exceed 2.3% on the average of the 3 flocks.

With two exceptions, all strains isolated were *C. jejuni* (Table I). Most of the strains showed typical biochemical behaviour, but ten did not grow in the presence of 1% glycine, one was resistant to nalidixic acid, one did not hydrolyse hippurate and the majority of the strains failed to grow in thiol medium containing 1% ox bile. With some exceptions, strains grew well and reduced both TTC and sodium selenite, while forming red or orange coloured colonies on blood agar. Ten out of the 14 TTC-negative strains did not grow on selenite agar, the remaining 4 grew well and reduced sodium selenite.

All the *C. jejuni* strains examined were resistant to superseptyl, trimethoprim, penicillin (penicillin G, oxacillin and methicillin), novobiocin and vancomycin, but were sensitive to all other antibiotics and chemotherapeutics tested, including erythromycin, streptomycin, oxytetracycline, neomycin, kanamycin, gentamicin, chloramphenicol, oleandomycin, polymyxin-B and furadantine.

With the tube agglutination test 34 out of the 41 *C. jejuni* strains examined could be assigned into one of six serogroups (Table II). Although we

Table I

Growth and biochemical characteristics of campylobacter strains isolated from laying hens and chickens

No. of strains	Catalase	Growth at 25 °C	Growth at 43 °C	1% glycine	H_2S lead acetate strip	TSI	Nalidixic acid 40 μg/ml	3.5% NaCl	0.1% TTC	0.1% Na selenite	1% ox bile	Hippurate hydrolysis
62	+	–	+	+ (–10)	+	–	– (+1)	–	+ (–14)	+ (–10)	– (+18)	+ (–1)*

* Only 22 strains were examined

Table II

Serogroup distribution
of *C. jejuni* strains isolated
from laying hens and chickens

Serogroups	Number of strains
1	7
2	1
3	7
4	9
5	8
6	2
Untypeable strains	7
Total	41

used unabsorbed sera, no major cross reactions were observed. Seven strains could not be classified with our sera.

Discussion

The results indicate that *C. jejuni* strains isolated from cases of avian campylobacter hepatitis, from intestinal contents of chickens or from contaminated chicken meat cannot be differentiated from one another based on the growth and biochemical characteristics of the isolates. Among our isolates only one was hippurate-negative *C. coli*, and another belonged to the nalidixic acid resistant thermophilic (NARTC) strains (Skirrow and Benjamin, 1980). Most *C. jejuni* strains reduced TTC and in this respect they differ from both *C. fetus* subspecies.

C. jejuni is considered to be a normal inhabitant of the intestinal tract of a wide variety of wild and domestic animals, including birds. Among the possible sources of infection for man, chicken meat is probably the most frequent one (Marjai et al., 1982; Blaser et al., 1983).

Based on heat-stable antigens Lauwers et al. (1981) assigned *C. jejuni* strains cultured from human faeces into fifty serotypes. Some strains isolated from cattle and dogs belonged to the same serotype to which also several human isolates were assigned (Lauwers et al., 1981). Using the tube agglutination test, we could classify our *C. jejuni* strains into six serogroups, but probably several further groups exist among them. The differences in heat-stable antigens of *C. jejuni* isolates from chicken may give an explanation for the reappearance of campylobacter hepatitis in the same flocks of laying hens.

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BIOCHEMICAL AND SEROLOGICAL CHARACTERIZATION OF *CAMPYLOBACTER* STRAINS ISOLATED FROM ABORTED BOVINE FETUSES

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Campylobacter strains isolated from aborted bovine fetuses were examined biochemically and serologically. Out of the 31 isolates, recovered from the stomach contents, lung and liver of 28 aborted fetuses, 29 proved to be *C. fetus* subsp. *venerealis*, the remaining two were *C. fetus* subsp. *fetus* and *C. jejuni*, respectively. The strains showed typical growth and biochemical characteristics and, apart from superseptyl, trimethoprim, penicillins, vancomycin and novobiocin, they were sensitive to all the other antibiotics tested.

With the tube agglutination test using heated antigens (10 min at 100 °C) all *C. fetus* strains tested could be assigned to serogroup 01 (A). The *C. jejuni* strain belonged to a separate serogroup, but showed serological relation to *C. jejuni* strains isolated from the faeces of dogs suffering from diarrhoea.

Keywords. *Campylobacter*, aborted bovine fetuses, biochemical and serological characterization.

Bovine genital campylobacteriosis (vibriosis) involving delayed conception, abortion and often infertility has a great economic significance in both dairy and beef cattle herds. The disease has been known for a long time, its occurrence has been reported in several countries (Moynihan and Stovell, 1955; Szabó, 1955; Clark, 1971; Smibert, 1978; Garcia et al., 1983). In the aetiology of the disease two biotypes of *Campylobacter fetus* (Florent, 1959) are involved, namely *C. fetus* subsp. *venerealis*, which corresponds to the former *C. fetus* subsp. *fetus* (Smibert, 1974), and *C. fetus* subsp. *fetus*, which was formerly named *C. fetus* subsp. *intestinalis* (Véron and Chatelain, 1973; Skerman et al., 1980). The natural habitat of *C. fetus* subsp. *venerealis* is the bovine reproductive tract, it causes enzootic abortion and infertility in cattle and is transmitted venereally. *C. fetus* subsp. *fetus* commonly occurs in the intestinal tract of sheep and cattle and can cause sporadic abortion and infertility in cattle and, more often, in sheep. Although it was supposed not to be transmitted venereally, recent reports indicate that this organism is able to colonize the reproductive organs of both heifers and bulls for more than a month (Agumbach and Ogaa, 1979). Therefore, its venereal transmission has to be considered. *C. fetus* subsp. *venerealis* has no public-health significance, but *C. fetus* subsp. *fetus* may be encountered in septicaemic diseases in man (Hallett et al., 1977).

Although artificial insemination is now a general practice in dairy herds in our country, recently sporadic, but increasing numbers of, campylobacter abortions have been observed. This paper reports the biochemical and serological examination of *Campylobacter* strains isolated from aborted bovine fetuses during the last two years in Hungary.

Materials and methods

Source of strains

Aborted bovine fetuses sent to Veterinary Investigation Centres for diagnostic purposes were examined. In case of suspected campylobacter abortions, samples from the stomach contents, lungs and liver were inoculated on blood agar, and on blood agar containing trimethoprim, vancomycin and polymyxin (Oxoid) (Skirrow, 1977) and growth-promoting supplement (George et al., 1978). The plates were incubated at 37 °C for 7 days under microaerophilic (6% O_2, 10% CO_2, 84% N_2) conditions. Out of 28 aborted fetuses, from 26 dairy cattle herds located in different parts of the country, 31 campylobacter strains were isolated.

Biochemical and antibiotic-sensitivity tests

Growth and biochemical characteristics, except H_2S production, triphenyltetrazolium chloride (TTC) and sodium selenite reduction, were examined in thiol medium (Difco) according to standard criteria (Véron and Chatelain, 1973; Smibert, 1974). For H_2S production, brucella broth (Difco) with 0.1% agar and with 0.02% cysteine, and TSI (Triple Sugar Iron) agar (Difco) were used. Growth in the presence and reduction of 0.1% TTC and of sodium selenite were tested on blood agar. Hippurate hydrolysis was examined as described by Hébert et al. (1982). All semisolid media were incubated in normal atmospheric conditions. Antibiotic sensitivity of 16 strains was examined with antibiotic-containing discs (Resistest, Human) on blood agar. For checking their growth, all isolates were inoculated on blood agar and into thiol medium, both containing the selective supplement (Oxoid) suggested by Lauwers et al. (1978).

Serological examinations

Twenty-four strains were examined with tube agglutination. Sera were produced in rabbits against *C. fetus* subsp. *venerealis* (O1) and *C. fetus* subsp. *fetus* (O2) (Mitscherlich and Liess, 1958). As antigens, freshly-grown strains washed off from blood agar plates with saline containing 0.1% formalin were used. Each rabbit received five rising doses (0.2, 0.5, 1.0, 1.5, or 2.0 ml) intravenously, at 3- to 4-day intervals. The rabbits were bled on day 7 after the

last injection. The end titre of both sera was 1 : 640. As antigen for the tube agglutination test, a bacterial suspension washed off with saline was used after heating at 100 °C for 10 min. Agglutination was read after 24 h at 37 °C.

Results

Campylobacter strains were cultured in all of our cases from the stomach contents of aborted fetuses. In two cases the same strain could be isolated also from the lungs and in one case from the liver of the fetus as well. All strains grew well, and almost always in pure culture on blood agar containing trimethoprim, vancomycin and polymyxin. Out of the 31 strains causing abortion, 29 belonged to *C. fetus* subsp. *venerealis*, the remaining two to *C. fetus* subsp. *fetus* and *C. jejuni*, respectively (Table I).

The antibiotic sensitivity of the isolates was uniform. All were resistant to superseptyl, trimethoprim, penicillins (penicillin G, oxacillin, methicillin), novobiocin and vancomycin, and all were sensitive to all other tested antibiotics or chemotherapeutics, including erythromycin, streptomycin, oxytetracycline, neomycin, kanamycin, gentamicin, chloramphenicol, oleandomycin, polymyxin-B and furadantine.

Contrary to our *C. fetus* subsp. *fetus* and *C. jejuni* strains, none of the *C. fetus* subsp. *venerealis* strains grew either on blood agar or in thiol medium containing the selective antibiotic supplement of Lauwers et al. (1978).

Table I

Characteristics of *Campylobacter* strains isolated from aborted bovine fetuses

Test	*C. fetus* subsp. *venerealis* (29 strains)	*C. fetus* subsp. *fetus* (1 strain)	*C. jejuni* (1 strain)
Catalase	+	+	+
43 °C	—	—	+
25 °C	+	+	—
H_2S (lead acetate strips)	—	+	+
Triple Sugar Iron (TSI) agar	—	—	—
1% glycine	—	+	+
3.5% NaCl	—	—	—
40 μg/ml nalidixic acid	+	+	—
1% ox bile	+/—*	+	+
0.1% triphenyltetrazolium chloride (TTC)	—	+	+
0.1% Na selenite	—	+	+
Hippurate hydrolysis	—	—	+

* 10 strains did not grow in thiol medium containing 1% bile

Out of the 24 strains examined in the tube agglutination test 18 *C. fetus* subsp. *venerealis* and the only *C. fetus* subsp. *fetus* gave agglutination up to the end titre with serum produced against *C. fetus* subsp. *venerealis* O1. Four strains could not be tested due to autoagglutination after heating. The only *C. jejuni* strain isolated in this series did not react with any sera against *C. fetus* but reacted up to 1 : 1280 with a serum produced against a *C. jejuni* strain (No. 89/1982), which had been isolated from the faeces of a dog suffering from diarrhoea.

Discussion

The results suggest that in Hungary campylobacter abortions in cows are most often due to *C. fetus* subsp. *venerealis*, but rarely *C. fetus* subsp. *fetus* and even *C. jejuni* strains might be involved. Growth characteristics and biochemical behaviour of the strains were typical, no intermediate types of *C. fetus* (Elazhary, 1968) were found. The *C. fetus* subsp. *venerealis* strains grow much slower than *C. fetus* subsp. *fetus*, and while the former do not grow on blood agar containing either 0.1% TTC or 0.1% sodium selenite, *C. fetus* subsp. *fetus* and *C. jejuni* strains do grow on 0.1% TTC medium and, due to reduction of TTC, their colonies are red.

For the selective isolation of campylobacters from bovine fetuses, blood agar containing trimethoprim, vancomycin and polymyxin as suggested by Skirrow (1977) proved to be very useful. On the other hand, the antibiotic supplement of Lauwers et al. (1978), suggested for selective isolation of *C. jejuni* from faeces, inhibits the growth of *C. fetus* subsp. *venerealis*; therefore, its use should be avoided when aborted bovine fetuses are examined.

Serologically all of our *C. fetus* subsp. *venerealis* strains tested belonged to serogroup O1 (Mitscherlich and Liess, 1958), either A (Morgan, 1959) or A1 (Berg et al., 1971), while the *C. fetus* subsp. *fetus* isolate belonged also to O1, but A2 (Berg et al., 1971; Dedié et al., 1977). The *C. jejuni* strain, based on its heat-stable antigen, belonged to a serogroup to which several *C. jejuni* strains isolated from diarrhoeic dogs can also be assigned (unpublished data).

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SIMPLE METHOD FOR OBSERVING MIGRATING LEUCOCYTES IN THE SKIN OF MIRROR CARP (*CYPRINUS CARPIO* L.)

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A special adaptation of Rebuck's skin window for the mirror carp (*Cyprinus carpio* L.) was developed. The method is based on insertion of a piece of a thin glass square (coverslip) intradermally. During an incubation of less than 24 h, migrating leucocytes attach to the glass square, where they can be stained for observation. It was demonstrated with *Mycobacterium phlei* bacteria that the glass squares can also be used for locally introducing substances which influence the activity of migrating leucocytes.

Keywords. Migrating leucocytes, method, mirror carp (*Cyprinus carpio* L.).

The methods applied in the studies on immunological properties of fish mostly originate from those developed for land animals. Much information has been gathered by direct application of these methods. However, adaptation has often been necessary to accommodate them to the special anatomical or functional characteristics of the fish. In this work, we tried to develop an adaptation of the in vivo leucocyte migration test by Rebuck's skin window (Rebuck and Crowley, 1955) for the mirror carp.

Materials and methods

Mirror carp of 20 to 150 g, originating from commercial fish ponds, were treated against ectoparasites and were kept in flow-through tanks at 1 kg fish/80 l water density with aeration.

In the skin of anaesthetized fish, a pocket of about 5 mm width and 7 mm length was prepared on the dorsolateral area of the anterior part of the body. The pocket opened caudally to prevent the water stream from reopening it (Fig. 1). The epithelium was cut, making a limited pressure move dorsoventrally, and a blunt injection needle was inserted into the slit and forwarded carefully under the Malpighi's layer of the epithelium, i.e. the stratum spongiosum of the corium (Szabó and Prigli, 1977), cranially.

The surface for adhesion of different cells migrating into the pocket was provided by a 3×3 mm sterile coverslip with even edges. The coverslips were prepared with the help of a ruler and a diamond glass-cutter. Five microlitres of sterilized 0.5% agarose solution in distilled water were dried on each piece

as to furnish it with a carrier for soluble stimulants or insoluble matters, e.g. carbon particles or bacteria, which stick to the agarose surface but get free in the skin pocket. Soluble substances, if heat-stable, are to be dissolved in the agarose solution; particulate matters suspended in distilled water can be dispersed on the dried film of agarose and dried again in sterile air. Gelatine is not suitable for the same purpose. In this paper, we publish about applying in the skin-pocket *Mycobacterium phlei* bacteria, which are simple to demonstrate for their special staining. A suspension of three days old culture of *M. phlei* on egg-yolk agar was made to give 0.1 absorbance at 525 nm. Five microlitres of the suspension were dispensed on glass squares precoated with agarose.

The square coverslips were placed into the skin-pocket after carefully lifting the free layers of the skin off. The agarose film was turned towards the body. Then, the epithelium was smoothed back. The glass squares were left in the skin-pockets for various durations indicated in the Results.

For microscopic analysis of migrating leucocytes, the glass squares were lifted away from the body by a pair of pincers, pulled off and gently rinsed in sterile 0.65% NaCl solution to eliminate excess cells and exudate. Then they were dipped into methanol for fixation, and were dried. Glass squares from several fish can be handled simultaneously, stuck on a slide with a vitreously transparent glue. Canada balsam was not suitable. Staining can be done as blood smears are stained. Our specimens were subjected to Ziehl–Neelsen, May–Grünwald–Giemsa staining and to the peroxidase test for neutrophils.

In situ histological examination of the migrating cells was done on materials fixed with 4% formalin and stained with haemalaun–eosin. For this, 24-h skin-pockets were cut around after the fish was killed, and were fixed. The glass squares were removed only after fixation.

The cells were denoted according to the nomenclature used by Ivanova (1983).

Results

We attempted unsuccessfully to include scaled carp because the skin-pocket could close due to the presence of scales. Channel catfish (*Ictalurus punctatus* Rafinesque) or the European catfish (*Silurus glanis* L.) of the same body mass range were also unsuitable for their epithelium tears easily.

The fish with glass squares in their skin showed no sign of excitement. A fish could have more than one skin-pocket at a time, thus, the cellular processes could be followed as a function of time. The skin-pocket itself closed so that the exudate inside formed a vesicle. Strongly antigenic matters like mycobacteria elicited subepithelial vasodilatation visible by the naked eye.

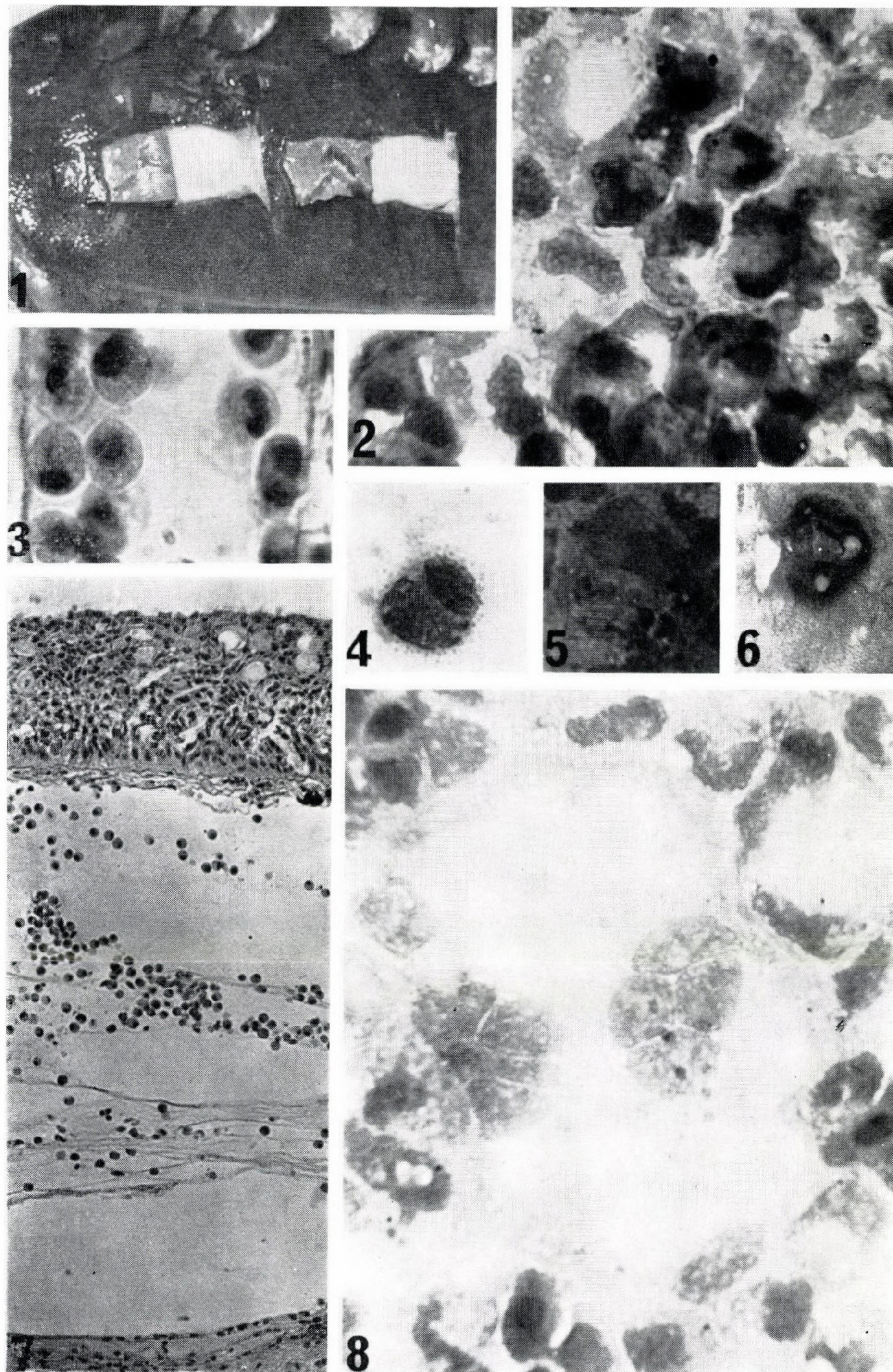


Plate I

Fig. 1. Dorsolateral area of the body of a mirror carp with two skin-pockets cut open. The free layers of the skin are reflected

Fig. 2. Continuous layer of leucocytes on the glass square after 24-h incubation in the skin-pocket at 18 °C. The group of darker cells consists of less differentiated neutrophils. May–Grünwald–Giemsa staining, ×780

Fig. 3. Young and ripe neutrophils in the exudate of the skin-pocket. A histological section. Haemalaun–eosin staining, ×780

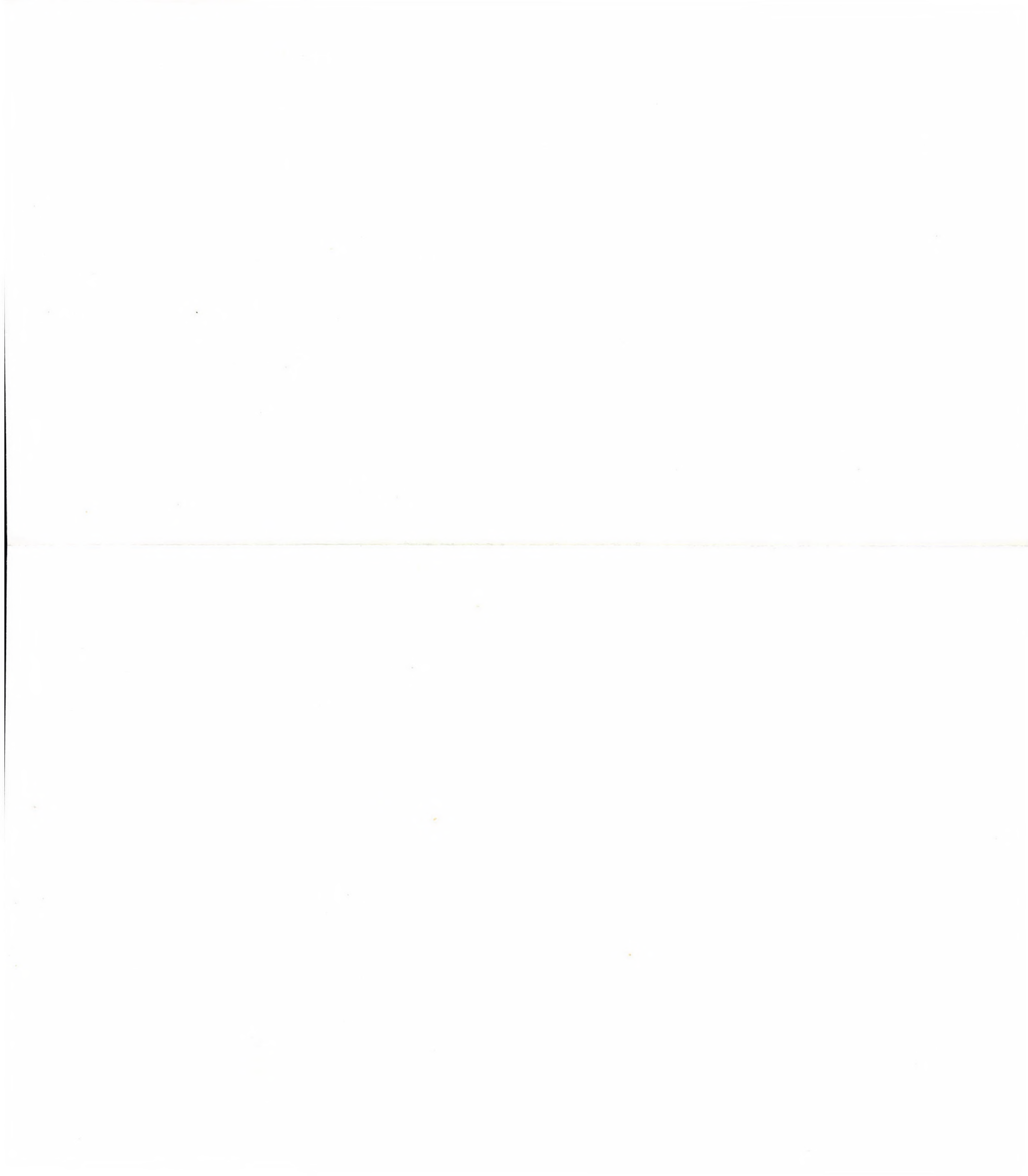
Fig. 4. Pseudobasophilic cell attached to the glass square. May–Grünwald–Giemsa staining, ×780

Fig. 5. Phagocytosed *M. phlei* bacteria and melanin particles in the macrophage, in situ on the glass square. Ziehl–Neelsen stain, ×950

Fig. 6. Two days old skin-pocket. Note the small necroses of the skin around the glass square

Fig. 7. Cross-section of a skin-pocket. Space between the epithelium–basal membrane–str. subepitheliale (above) and the corium (below) is filled with an exudate containing leucocytes. A histological section. Haemalaun–eosin staining, ×63

Fig. 8. A giant cell on the glass square. May–Grünwald–Giemsa staining, ×950



Empty skin-pockets healed per primam, leaving behind a discolouration of the affected skin surface. Fish having thin and weakly-built skin were unsuitable for preparing pocket. Sometimes, when the skin-pocket was not long enough or got torn, the glass square got lost.

Comparison of glass squares with and without agarose film on them showed that no significant difference in the amount and quality of the migrating leucocytes was caused by the film. Difference was found in how many cells remained attached on the glass square after rinsing in saline. The agarose film kept a greater part of the attached cells than the glass surface. The well-dried agarose film did not tend to peel off. Careless handling could cause creases of the film.

An experimental group of fish was held at 10 °C, another at 16 °C. Each of the 24 fish in each group had four skin-pockets that were emptied in every second hour, one by one. No cells attached to the agarose film during the first two hours. In fish kept in 10 °C water, the first solitary myeloblast cells, up to 20, appeared in the pockets of six fish by the end of the 4th hour. A 6-h incubation resulted in 12 fish with myeloblast cells, some of which started to proliferate. After eight hours, myeloblast cells and more differentiated myeloid forms were on the agarose film of 18 specimens. The myeloblast cells formed groups of young myeloid cells in eight fish (Fig. 2).

In the fish group kept at 16 °C, the same process advanced at a faster rate. Cell migration took place within the first two hours, and groups of young myeloid cells appeared by the end of the 4th hour.

The individuals of fish showing the most advanced cell migration and proliferation were not the same at the subsequent samplings.

A 24-h period at 18 °C allowed great masses of ripe neutrophils to appear in the skin-pocket of ten from fifteen carp (Fig. 3). The rest of the squares also had an almost continuous lawn of cells. About 94% of the cells belonged to the neutrophil line; about 4% were macrophages and about 2% pseudobasophilic (Fig. 4) and unidentified cells. Around a third of the neutrophils were ripe. Islets of young cells surrounding blast-type myeloid cells represented around another third. Older neutrophils started to vacuolize and some melt together into giant cells of two or more nuclei (Fig. 8). These nuclei lined side-by-side along the membrane, less frequently clumped together in the centre. There were disrupted neutrophils, too. Lymphocytes were rare. Red blood cells usually occurred in a limited number. There were no eosinophils present.

In a further experiment, glass squares carrying *M. phlei* were applied to the skin-pockets of 14 carp at 18 °C. Each had a contralateral control counterpart. After 24 h of incubation, a well-expressed swelling with capillary dilation was observed in the skin-pockets containing bacteria. Examination of the glass squares proved that hardly any (around 6%) of the bacteria remained

on the surface of the squares, and even these were phagocytosed by macrophages (Fig. 5).

Histological examination revealed a thinning of the epithelium outside the skin-pocket, presumably a result of insufficient circulation. Cells similar to those present on the glass squares could be seen in the tissues surrounding the skin-pocket and in the cavity (Fig. 7). The cells which were in connection with the glass square during fixation had a flat side. *M. phlei* induced a larger amount of cells to migrate and/or proliferate. No specific deviation was noted concerning the types of cells as compared to the cells in bacterium-free skin-pockets. Histological sections also demonstrated an ample exudation in and around the skin-pocket as well as an ectasy of capillaries.

Discussion

Rebuck's skin window (Rebuck and Crowley, 1955) provides a simple tool for visualization of the first cellular events of the defence mechanism. This test is done on the forearm of humans. First, an erosion is made to the depth of the dermal-epidermal junction, then a glass coverslip is pressed slightly to the surface of the abrasion for optional duration from 2 to 24 h. The coverslip can also be exchanged at regular intervals. The cells attached to the coverslip are then stained with May–Grünwald–Giemsa.

The difficulty of keeping animals living in water free of microbial contamination and the problem of how to fasten the coverslip to the fish were overcome by preparing skin-pocket. It does not require more than one minute and does not produce considerable bleeding. Relatively few red blood cells were found in the skin-pockets. The glass squares can be changed for new ones in the skin-pockets. However, after a period longer than one day one has to think about malnutrition of the tissues outside the skin-pocket, depending on temperature because degeneration combined with the neutrophil activity inside may lead to small necrotic ulcers and infection of the skin-pocket (Fig. 6).

Rebuck's skin window gives an insight into cell migration but is not suitable for quantitative characterization of this process. Normally, however, one can find in the first 6 to 8 h neutrophils which are gradually replaced by macrophages. The latter will predominate by the 24th h.

We could not characterize the cell migration in the skin-pockets statistically. This is the reason why we publish approximate values of relative amounts of cells. The first cells to appear were blast-type, peroxidase-positive cells that assumedly generated groups of young cells by proliferation, and by 24 h at 18 °C a continuous layer of mostly young and ripe neutrophils covered the glass square. Macrophages were not present at a high ratio, so no process

in their overtaking could be stated. The phenomena were characteristic of fish in the respect of the temperature-dependence and presence and activity of blast-stage cells. These cells let us have a look, besides the migration, at the proliferation of the neutrophils as well.

The skin-pocket method combined with the carrier agarose layer can possibly be used in testing inhibitory and cytotoxic agents.

In the study of neutrophil activity *per se* in carp, skin-pockets may again have a wide range of possibility for use.

The question whether Rebuck's skin window induces a foreign body reaction rather than the early phase of the neutrophil defence has remained open. The same theoretical problem may be raised in the case of the skin-pockets, all the more so because our knowledge about the cellular defence in fish is much less than that of human immunology. In our view, skin-pocket of mirror carp may be an in vivo method easily applicable in any laboratory, perhaps contributing to the answer to the above question, too.

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BIOLOGY AND HISTOPATHOLOGY OF *THELOHANELLUS HOVORKAI* ACHMEROV, 1960 (MYXOSPOREA, MYXOZOA), A PROTOZOAN PARASITE OF THE COMMON CARP *(CYPRINUS CARPIO)*

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Thelohanellus hovorkai, a protozoan introduced in Hungary from the Far East, is a frequent parasite of one- and two-summer common carp reared in fish ponds. The plasmodia are demonstrable most frequently in the summer months, in the buccal tissues of the head of fry. In one-summer common carp, plasmodia occur more frequently on the swimbladder serosa. A certain seasonality can be observed in the occurrence of *T. hovorkai;* namely, infection appears in early summer and disappears by the end of summer. The first developmental stages were demonstrable in 4 weeks old fry; in 6 to 8 weeks old fish mostly cysts containing only spores occurred. *T. hovorkai* is a typical connective-tissue parasite; its development is always associated with the dense connective tissue. The cysts of *T. hovorkai* are 0.3 to 0.5 mm in diameter, are surrounded by one or two rows of connective-tissue cells, and most frequently sit on ligaments, fasciae or on the adventitia constituting the walls of blood vessels.

Keywords. *Thelohanellus hovorkai*, Protozoa, parasite, biology, histopathology, common carp *(Cyprinus carpio)*.

In an earlier paper (Molnár and Kovács-Gayer, 1981–82) we reported the occurrence in Hungary of two common carp parasite *Thelohanellus* species introduced from the Far East, *Thelohanellus nikolskii* Achmerov, 1955 and *T. hovorkai* Achmerov, 1960. Of these two species, the biology and histopathology of *T. nikolskii* were described in detail by Molnár (1982), and its ultrastructure by Desser et al. (1983).

In the present paper our observations made on the biology, pathomechanism and tissue specificity of *T. hovorkai* during the dissection and histological examination of common carp reared in pond farms are reported.

Materials and methods

Common carp from the fry-rearing and rearing ponds of different Hungarian fish farms were used. The investigations were started in 1980, when the parasite was first demonstrated histologically in common carp submitted for routine laboratory examination. In 1981 and 1982 mainly two-summer fish were examined, but in 1982 to 1984 the investigations were extended to the fry as well. At two-week intervals samples were taken from the fish ponds that had been found infected in earlier years, and the sampled fry were

examined by dissection continuously. Results on two-summer fish came from the processing of fish sent for routine laboratory examination.

Infection was monitored by light-microscopic examination of squash preparations made from the organs and smaller parts of the fish (the organs and body parts were squashed between glass-plates). When spores or cysts were present, the infected organs were fixed for histological examination. In 1983 and 1984, 2- to 3-cm-long fry from consistently infected fish ponds were fixed in their entirety, without previous examination.

For histological fixation, 10% neutral formalin or Bouin's solution was used. After embedding in paraffin, 4 μm thick sections were made from the organs, and from fry cleaved in the longitudinal plane of the body. The sections were stained with haematoxylin and eosin, and according to Farkas–Mallory's and van Gieson's technique.

Results

Observations

In a histological section, a *Thelohanellus hovorkai* cyst was first observed in 1980, on the swimbladder serosa of a two-summer common carp. In 1981 further cysts were found in two-summer fish in the subserosal connective tissue of the swimbladder, and such cysts occurred also in the connective tissue of the gill arch. In that year cysts were found on the swimbladder in native state, and the examination of spores obtained from the cyst rendered possible a precise definition of the parasite species. In 1982 it became clear that *T. hovorkai* was a frequent parasite of certain fish farms; intensive infection occurred mainly in fry. Investigations conducted between 1982 and 1984 revealed that the first cysts occurred already in one-month-old, 2 to 2.5 cm long fry, and in 6 to 8 weeks old fish even spore-containing cysts were demonstrable. In some stocks the infection rate reached 80 to 90%. In the majority of cases, *T. hovorkai* infection occurred jointly with *T. nikolskii* infection. The clinically apparent *T. nikolskii* infection was pathognomic since it occurred synchronously with *T. hovorkai* infection, which could not be diagnosed solely by exterior examination. By native examination *T. hovorkai* infection was easiest to demonstrate from the head of the fish. Under a stereomicroscope the small, pinhead-sized cysts 0.3 to 0.5 mm in diameter could be released from the buccal tissues with a dissecting needle. The cysts contained relatively few, most frequently 200 to 400, spores. As a rule, infection ceased to exist by the end of August. In September only a few scattered spores were found in the melanomacrophage centres of the kidneys and spleen. No clinically apparent disease or deaths were observed in intensively infected stocks either.

T. hovorkai infection did not occur in grasscarp, silver carp and bighead stocks kept in the infected fish ponds.

Histological studies

In fry, *T. hovorkai* cysts occurred most frequently in the periorbital loose connective tissue (Fig. 1) and around the nose; however, cysts were of frequent occurrence also in the operculum, among the muscles bordering the gill-opening (Fig. 2) and in the perirenal connective tissue. Less frequently, solitary cysts were demonstrated also in the gill arch, among the tail muscles, and in the connective tissue surrounding the vertebral column. In two-summer fish, cysts were found in three locations: most frequently in the subserosal connective tissue of the swimbladder and in the compact connective tissue covering the gill arch cartilage. However, on one occasion a cyst containing numerous spores was found under the serosa of the extrahepatic pancreas lying on the intestinal wall, embedded in the pancreatic substance.

The plasmodia always started to develop among connective-tissue cells, irrespective of the organ in which they developed. They showed pronounced affinity for the compact connective tissue; therefore, even the cysts situated in the loose connective tissue frequently sat on ligaments, fasciae, or on the adventitia constituting the wall of blood vessels (Figs 1, 4 and 5). Among the muscles the connective tissue separating muscle fibres, whereas in the case of the serous membranes the connective tissue lying beneath the coelothel of the serosa was their site of establishment. In parenchymal organs no plasmodia occurred except those lying beneath the serosa covering these organs; however, after the disruption of cysts that had developed in other parts of the body, one or two spores were frequently demonstrated in the melanomacrophage centres of the above organs. *T. hovorkai* plasmodia were surrounded by a rather thin capsule consisting of one or two rows of connective tissue cells. The youngest plasmodia observed by us were 16 to 20 μm in diameter and approximately round in shape (Fig. 3); within them, a 4-μm-thick ectoplasm and an endoplasm constituted by 12 to 20 cells were distinguishable. The plasmodium was surrounded by connective-tissue cells having relatively large nuclei. The plasmodia showing a more advanced stage of development and containing pansporoblasts and immature spores were also spherical in shape. Within the cyst 80 to 120 μm in diameter, the 8 to 10 μm thick, eosinophilic ectoplasm and the endoplasm were well-distinguishable (Fig. 4). The cysts, attached with one pole to the adventitia of blood vessels or to other compact connective tissues, were separated from the surrounding loose connective tissue by a thin capsule. The mature, spore-containing cysts were mostly oval or ellipsoidal in shape and reached a size of $160\text{–}200 \times 70\text{–}110$ μm in fry (Fig. 5) and even 300×140 μm in two-summer common carp. Their ectoplasm disappeared, the connective-tissue cells covering the cyst became flattened. Beside the loose connective tissue, the cyst wall was frequently contiguous with the coelothel, muscle cells or parenchymal cells; however, the compact

connective tissue indicating the origin was present on one of the poles of the cyst also in these cases (Fig. 5).

Discussion

The present studies indicate that *T. hovorkai* is a common carp parasite widespread in Hungary; it is at least as frequent as the fin parasite *T. nikolskii*. Since the plasmodia of *T. hovorkai* are relatively small and develop in the tissues, without microscopic examination it is difficult to diagnose the infection, which frequently remains unnoticed. Obviously, the parasite was introduced into Hungary from the Far East, similarly to *T. nikolskii* (Molnár and Kovács-Gayer, 1981/82) and has become widespread among common carp cultured in pond farms. According to our investigations, the infection is most frequent in the young fry; however, it occurs in two-summer fish as well. *T. hovorkai* is a connective-tissue parasite; thus, it may occur in all body parts which are rich in connective tissue. In fry, the most frequent location of the parasite is the connective tissue separating the muscles of the head and those around the pharynx, while in older fish the parasite was demonstrated in the connective tissue of serous membranes and gill arches most frequently. A more precise localization of the infection in older fish was hampered by the fact that in fry the location was determined by processing and examining all organs, whereas in two-summer fish organs less important from the aspect of routine examinations were not always studied.

Therefore, it cannot be excluded that in older fish infection occurs also in places other than the gills and serous membranes. The development of *T. hovorkai* shows a yearly cycle pattern. Young plasmodia appear in the fish in June or July. Spores are formed within the plasmodia after a developmental period of 4 to 6 weeks, depending on the temperature. In 6 to 8 weeks old fish mainly spore-containing cysts are found. Young cysts are surrounded by connective-tissue cells rich in plasm; later on, parallel to the growth of plasmodia, these cells become flattened and form a thin ring of 1 or 2 cell rows. After the disruption of cysts the 200 to 400 spores get into the neighbouring connective tissue, from where the lymph or blood circulation transports them to various organs. The excretion of spores to the outworld is obviously similar to that described for the muscle parasite *Myxobolus cyprini* by Molnár and Kovács-Gayer (1985); i.e. the spores are expulsed from the capillaries of the gills, skin, renal tubules and intestine to the outworld through local necroses. At the same time, spores stuck and encapsulated in the cyst, or destroyed in the melanomacrophage centres of the spleen and kidneys, were found also in *T. hovorkai* infection.

In the Far East, besides *T. hovorkai* several other *Thelohanellus* spp. parasitize the common carp *(Cyprinus carpio)*. Of these, Achmerov (1960)

reported *T. nikolskii* from the fins, *T. dogieli* from the skin, *T. hovorkai* from the peritoneum, *T. acuminatus* from the gill lamellae, and *T. amurensis* from the liver of the common carp. Japanese authors widened the circle of these parasites. Hoshina and Hoshoda (1957) reported the species *T. cyprini* from the fins, while Egusa and Nakajima (1981) *T. kitauei* from the intestinal wall. Although Shulman (1966) considered the majority of the species to be synonymous with *T. dogieli* and *T. furmanni*, based upon spore morphology and the pronounced organo-specificity today it is unquestionable that only *T. cyprini* can be regarded as a synonym of *T. nikolskii*, while the validity of the remaining species seems to be proved. The extraordinary organo-specificity of *Thelohanellus* spp. was first described by Achmerov (1955). Later on, his observation was supported by the results of Molnár (1982) and Molnár and Kovács-Gayer (1981–82) on *T. nikolskii* and *T. hovorkai*. The development of *T. hovorkai* within the fish indicates that in the case of *Thelohanellus* spp. the term "tissue-specificity" should be used instead of organo-specificity.

Although, similarly to Achmerov (1960), we also found *T. hovorkai* first on the serosa, more detailed investigations have revealed that this species occurs in all organs rich in connective tissue. *T. hovorkai* is a connective-tissue parasite, as opposed to the cartilage parasite *T. nikolskii*, whose development is associated with perichondrial cells (Molnár, 1982), or to *T. kitauei* whose typical site of development is the intestinal mucosa. In addition to the tissue-specificity of *Thelohanellus* spp. parasitizing the same host, pronounced anatomical differences existing in spore morphology ensure an accurate species identification. Similarly, the various species are characterized by different host responses. *T. hovorkai* spores, differing from the spores of *T. nikolskii* in their considerably larger polar capsules, develop in relatively small cysts containing significantly fewer spores than those of *T. nikolskii*. In *T. hovorkai* infection, the host response developing around the small cysts and restricted to a connective-tissue capsule consisting of a few cell rows, can be considered negligible as compared to the thick, cartilageous and connective-tissue capsule developing around *T. nikolskii* plasmodia.

Unfortunately, no conclusions on the pathogenicity of *T. hovorkai* can be drawn from the present studies. *T. hovorkai* seems to be a species of low pathogenicity; since it is located in less vital organs, it presumably does not cause deaths among the fish. However, because of its regular and consistent occurrence primarily in fry, its role in decreasing the host's resistance should not be neglected.

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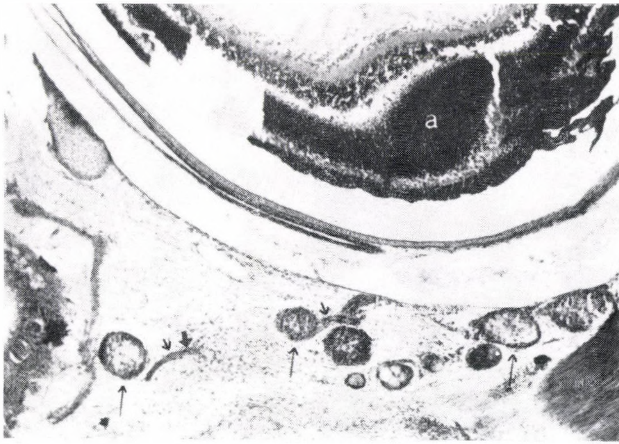

Fig. 1. Thelohanellus hovorkai cysts in the periorbital connective tissue. Orbital cavity (a), cysts (↑), dense connective tissue (↑). Haematoxylin–eosin, ×100

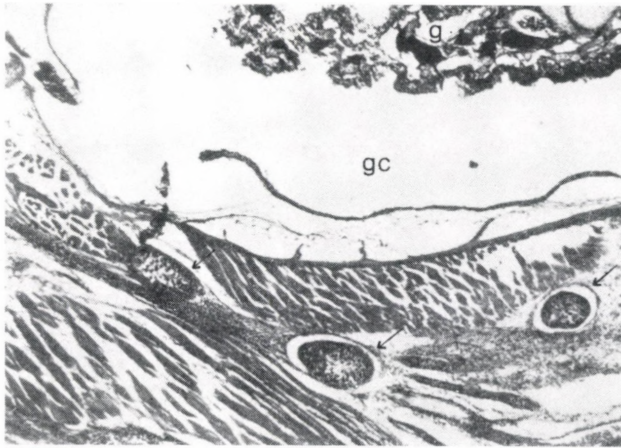

Fig. 2. Thelohanellus hovorkai cysts among the muscle bundles neighbouring the gill cavity. Gill cavity (gc), gill (g), cysts (↑). H. and E., ×100

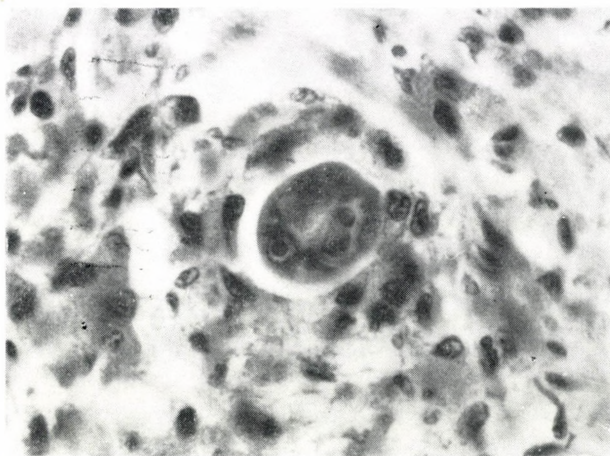

Fig. 3. Young *Thelohanellus hovorkai* plasmodium in the loose connective tissue of the head of a common carp fry. H. and E., ×1200

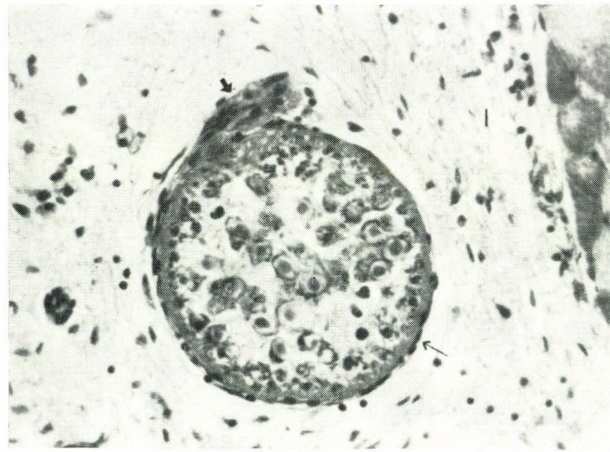

Fig. 4. Developing plasmodium containing young spores in the loose connective tissue. Loose connective tissue (1), dense connective tissue (↑), connective tissue cells constituting the capsule of the cyst (↑). H. and E., ×400

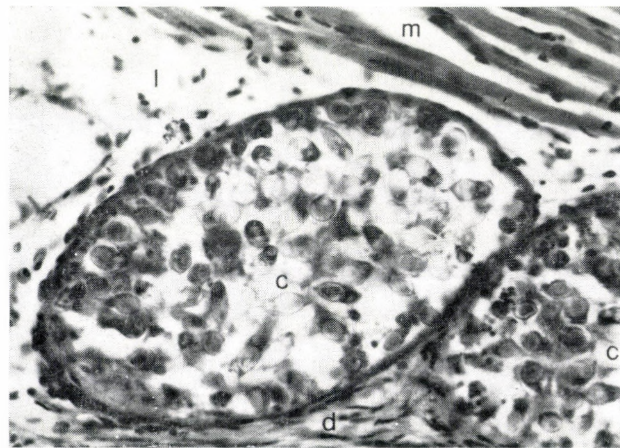

Fig. 5. Thelohanellus hovorkai cysts containing mature spores in the intermuscular connective tissue of common carp fry. Cysts (c), loose connective tissue (1), dense connective tissue (d), muscle fibres (m). H. and E., ×400

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HAEMONCHUS CONTORTUS: LIPID BIOSYNTHESIS FROM ^{14}C-LABELLED PALMITIC ACID AND SODIUM BICARBONATE

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Adult *Haemonchus contortus* (Rud., 1803) was investigated for its ability to synthesize lipids from simple ^{14}C-precursors. It was shown to have extensively active mechanisms for synthesizing all classes of complex lipids present, including free cholesterol from HCO_3^- and ^{14}C-palmitic acid. Insignificant amounts of lipids were synthesized from HCO_3^- relative to that from palmitic acid. With both the precursors, there is more of nonpolar (NP) than polar (P) lipid synthesis, the NP/P ratio being 1.258 in case of HCO_3^- and 1.917 in case of palmitic acid. Among NP lipids, free fatty acids and triacylglycerols were synthesized to a greater extent than the other components. Significantly, the synthesis of hydrocarbons and pigments was more intensive from HCO_3^- than from palmitic acid. As regards the P lipids, most of the label was incorporated into phosphatidyl choline (PC) and phosphatidyl ethanolamine (PE) irrespective of the precursor employed, the PC/PE ratio being 1.237 in case of HCO_3^- and 1.313 in case of palmitic acid. Phosphatidyl serine was synthesized only to a small extent from palmitic acid relative to the synthesis from HCO_3. Also, more label from HCO_3 than from palmitic acid was incorporated into cerebrosides.

Keywords. *Haemonchus contortus*, lipid, biosynthesis, palmitic acid, sodium bicarbonate.

Haemonchus contortus (Rud., 1803) is the most pathogenic nematode parasite of sheep, goats and other ruminants. It has attracted considerable attention of investigators in different parts of the world (see Sood and Kapur, 1982). However, no attempt has been made to study its lipid metabolism, although its carbohydrate metabolism has been studied extensively (Ward, 1974; Ward and Huskisson, 1978; Ward and Huskisson, 1980; Ward, 1982; Kaur and Sood, 1982).

Nematodes are dependent on carbohydrates for their survival; lipid biosynthesis is less important as a means of energy storage. However, lipids are not solely used as energy sources. Lipids are incorporated into eggs and are important constituents of membranes which turn over constantly. All these facts do not eliminate the possibility of lipid biosynthesis in nematodes. Also, carbon from linoleic acid is incorporated into various membrane structures of adult *Schistosoma mansoni* (Rumjanek and Simpson, 1980). Adult *H. contortus* has been shown to be capable of synthesizing fatty acids, complex lipids and simple precursors such as acetate and glucose (Kapur and Sood, 1984*a*). However, there are only a few reports in parasitic helminths regarding the impor-

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tance of long-chain fatty acids in lipid biosynthesis. These include studies on *Ascaris* (Jezyk, 1968; Greichus and Greichus, 1970), *Hymenolepis diminuta* (Lumsden and Harrington, 1966; Jacobsen and Fairbairn, 1967; Overturf and Dryer, 1968), *Caenorhabditis briggsae* and *Panagrellus redivivus* (Rothstein, 1970) and *Spirometra mansonoides* (Meyer et al., 1966*a*). The role of long-chain fatty acids in the lipid biosynthesis of *H. contortus* has not been studied at all.

Fixation of CO_2 by adult *H. contortus* has been well established (Ward and Huskisson, 1980). Products formed in the CO_2 fixation pathway (Saz and Lescure, 1965), e.g. acetyl CoA, are important precursors involved in lipid biosynthesis. Acetyl CoA also comes from pyruvate. Also, succinate (product of CO_2 fixation) may be incorporated into glycerol and the amino or sugar portion of phospholipids. Propionate is also involved in fatty acid biosynthesis. However, there is no direct report regarding the importance of CO_2 in lipid biosynthesis.

The present studies were undertaken to investigate the metabolic role of long-chain fatty acids (palmitic acid) and CO_2 in lipid biosynthesis in adult *H. contortus*.

Materials and methods

Adults of *H. contortus* were collected from the abomasa of goats slaughtered at local abattoirs. Adhering materials were removed by washing in saline. 1-C^{14}-palmitic acid (specific activity, 21.1 mCi/mmol) and sodium C^{14}-bicarbonate (52 mCi/mmol) were procured from the Isotope Division of Bhabha Atomic Research Centre, Bombay, India.

Incubations. The incubation medium was a modified Tyrode's solution (Ward, 1974). Ten ml of the incubation medium was transferred to each of two flasks. CO_2 was passed through one of the flasks and 12.5 μCi of 1-^{14}C palmitic acid was added. Before added, palmitic acid was converted to its potassium salt by saponification with KOH. In the other flask (without CO_2), 12.5 μCi of sodium ^{14}C-bicarbonate was added. The final pH of each was adjusted to 6.6 $\pm$ 0.1. Worms 500 mg in weight were transferred to each flask, immediately after their recovery from the host. The flasks were incubated at 30 $\pm$ 1 °C in a metabolic shaker for 4 h.

After completion of the incubation period, a few drops of chloroform : methanol (2 : 1 v/v) were added to each flask in order to stop the incorporation. Media were poured out of the flasks and the worms washed several times with distilled water.

Extraction of lipids. Worms of the above groups were processed separately for the extraction of lipids. They were blotted dry and homogenized in chloroform : methanol (2 : 1 v/v) in a glass hand-homogenizer. Extracts were

kept on a shaker for 3–4 h and filtered by suction through a Grade 3-sintered glass funnel. Residues were reextracted thrice with chloroform : methanol (2 : 1 v/v). All the lipid extracts were pooled. Water-soluble impurities were removed by subjecting the crude lipid extract to washing (Folch et al., 1957). Chloroform phases containing lipids were stored at —20 °C until the analyses were complete.

Fractionation of lipids. Total lipids were separated into polar and nonpolar lipids by the method of Nichols (1964). Further fractionation of polar and nonpolar lipids was achieved by unidimensional thin-layer chromatography, on silica gel G plates (250 μm thick). For the fractionation of polar lipids, the chromatograms were developed in a solvent system of chloroform : methanol : water (65 : 25 : 4 v/v). Nonpolar lipids were fractionated by developing the chromatograms in petroleum ether : diethyl ether : acetic acid (80 : 20 : 1 v/v). After completion of development, the chromatograms were air-dried.

Identification of lipids. Various classes of polar and nonpolar lipids were identified by cochromatography with authentic standards and by the use of specific spray reagents (Kapur and Sood, 1984*b*). Once the identification was confirmed, silica gel from each spot was transferred directly into scintillation vials, for measuring radioactivity.

Measurement of radioactivity. Aliquots of total, polar and nonpolar lipids were taken into scintillation vials and the solvent evaporated at low temperature. Radioactivity was measured using a β-scintillation spectrometer. The scintillation fluid used for the total and nonpolar lipids had the following composition: 4 g PPO (2,5-diphenyloxazole), 200 mg POPOP (1,4-bis/5-phenyloxazolyl/benzene) and the volume was made up to one litre with toluene. For polar lipids, Bray's (Bray, 1960) scintillation fluid was used with the following composition: 4 g PPO, 200 mg POPOP, 60 g naphthalene, 20 ml ethylene glycol, 100 ml methanol, and the volume was made up to one litre with dioxan. In each vial, 10 ml of the appropriate scintillation fluid was added.

Results

Results of incorporation of ^{14}C into total, polar and nonpolar lipids are given in Table I. Only a small amount of label from sodium bicarbonate was incorporated into *total* lipids, as compared to that from palmitic acid. With both precursors, there is more of nonpolar than polar lipid synthesis, the NP/P ratio being 1.258 in case of HCO_3^- and 1.917 in case of palmitic acid.

Results of incorporation of label into various fractions on *nonpolar* lipids are given in Table II. In both cases, there was least incorporation of label into sterols. Most of the radioactivity was incorporated into free fatty acids

Table I

Incorporation of radioactivity into total, polar and nonpolar lipids of *H. contortus* (Rud., 1803)

Precursor	Lipids*			NP/P
	Total	Polar (P)	Nonpolar (NP)	
Sodium bicarbonate-^{14}C	14.44 (1.25)	6.39 (0.55)	8.05 (0.70)	1.26
Palmitic acid-1-^{14}C	460.19 (98.24)	157.73 (33.67)	302.45 (64.56)	1.91

Values are expressed/g of fresh tissue. Figures in parentheses indicate nmols of the radioisotope incorporated.

* $dpm \times 10^{-4}$

and triacylglycerols. Incorporation of label from palmitic acid into hydrocarbons and pigments was insignificant.

Results of incorporation of label into various fractions of *polar* lipids are given in Table III. It is clear that the order of incorporation from bicarbonate was somewhat different from that of incorporation from palmitic acid.

Table II

Incorporation of radioactivity into various fractions of nonpolar lipids of *H. contortus* (Rud., 1803)

Precursor	MG	DG	Sterol	FFA	TG	S. esters	Hyd. + pig.
Sodium bicarbonate-^{14}C	4.7	6.0	3.66	52.30	16.7	5.8	10.8
Palmitic acid-1-^{14}C	3.3	6.6	2.0	57.8	23.9	5.4	0.8

Results are expressed as the relative percentage of incorporation of total nonpolar lipids. MG: monoacylglycerols; DG: diacylglycerols; FFA: free fatty acid; TG: triacylglycerols; S. esters: sterol esters; Hyd. + pig.: Hydrocarbons + pigments

Table III

Incorporation of radioactivity into various fractions of polar lipids of *H. contortus* (Rud., 1803)

Precursor	PI	PS	Sph. + LPC	LPE	PC	PE	Cereb. I	Cereb. II
Sodium bicarbonate-^{14}C	10.9	8.1	6.7	4.9	24.4	19.7	15.0	10.0
Palmitic acid-1-^{14}C	3.2	0.7	5.4	7.7	41.3	31.5	4.3	5.7

Values are expressed as the relative percentage incorporation of total polar lipids. PI: phosphatidyl inositol; PS: phosphatidyl serine; Sph. + LPC: sphingomyelin + lysophosphatidyl choline; LPE: lysophosphatidyl ethanolamine; PC: phosphatidyl choline; PE: phosphatidyl ethanolamine; Cereb.: cerebrosides

Discussion

In the present studies, in *H. contortus* more label was incorporated into nonpolar (NP) than polar (P) lipids, regardless of the precursor employed. This observation is in good agreement with the findings in *A. lumbricoides* (Jezyk, 1968), *H. diminuta* (Lumsden and Harrington, 1966; Jacobsen and Fairbairn, 1967), *Dirofilaria immitis* and *Fasciola hepatica* (Barrett and Körting, 1976). On the contrary, with acetate and glucose, more label was found in P than NP lipids of *H. contortus* (Kapur and Sood, 1984*a*). Thus, it can be concluded that the NP/P ratio does not merely reflect the mass ratio of these two classes of lipids, rather it may indicate their specific metabolism. Further, in *S. mansonoides*, oleic acid is incorporated in equal amounts in P and NP lipids (Meyer et al., 1966*a*). Disparity in these findings may be due to the fact that only a small amount of the label is incorporated into total lipids.

Since the greatest part of the label from palmitic acid was incorporated into FFA, it is postulated that, as in vertebrates, palmitic acid is first converted to acetate, which is further incorporated into various components via its incorporation into fatty acids. Only a part of palmitic acid is converted directly to acylglycerols. Therefore, fatty acids being nonpolar, more activity is found in NP lipids. Further, since NP lipids are highly labelled compared to P lipids, and also among NP lipids, triacylglycerols are the most intensely labelled, it is postulated that NP lipids are the precursors of P lipids, through the transfer of fatty acids to phospholipids.

Regarding the incorporation of carbon from HCO_3^-, the extramitochondrial system for *de novo* synthesis of fatty acids requires HCO_3^- (as a source of CO_2) for the initial reaction, i.e., the carboxylation of acetyl CoA to malonyl CoA. $^{14}CO_2$ is rapidly incorporated into succinate and propionate in *A. lumbricoides* eggs (Saz and Lescure, 1965). *Ascaris* muscle forms succinate anaerobically by means of CO_2 fixation into pyruvate (Saz and Vidrine, 1959). Succinate serves as a precursor of propionate, which in turn is utilized for the synthesis of odd-numbered fatty acids. Disappearance of HCO_3^- from incubation media containing *Nematodirus* spp. and *Ascaridia galli* was reported, thus suggesting CO_2 fixation (Rogers and Lazarus, 1949). Also *Heterakis gallinae* has been demonstrated to be capable of fixing radioactive CO_2 into succinate, by using $NaH^{14}CO_3$ (Fairbairn, 1954, 1960). CO_2 was also found to be essential for the incorporation of propionate into lipids of *A. lumbricoides* (Harris, 1968). In the present studies, only trace amounts of label from bicarbonate relative to palmitic acid were incorporated into total lipids. However, the rate of incorporation of labelled precursor does not indicate the actual rate of synthesis in terms of weight of lipid synthesis/g tissue/unit time (Overturf and Dryer, 1968). The exact amount of lipid synthesis cannot be determined unless the dilution of exogenous substrates with endogenous substrates from the metab-

olic pool is determined. However, this cannot be done from the present data. Also, it is possible that the availability of CO_2 in gaseous form would increase fixation. Therefore, further studies need to be carried out employing $^{14}CO_2$ in gaseous form.

Biosynthesis of cholesterol requires molecular oxygen, and *H. contortus* has been shown to be capable of high rates of respiration at low PO_2 (Rogers, 1949). Also, mitochondria from the oviduct of *H. contortus* resemble those from highly respiring rat brown adipose tissue (Ward, 1982). Therefore, it is not surprising that adult *H. contortus* is capable of sterol biosynthesis from palmitic acid and HCO_3^- as reported in the present studies. Also, sterol biosynthesis has been reported from acetate in *D. immitis* (Turner and Hutchison, 1979) and *A. lumbricoides* (Beames et al., 1967).

Incorporation of label from palmitic acid into acylglycerols is in the order: triacylglycerols $>$ diacylglycerols $>$ monoacylglycerols as also reported in *H. diminuta* (Overturf and Dryer, 1968) and *A. lumbricoides* (Jezyk, 1968). This is in contrast to the findings on *D. immitis* (Turner and Hutchison, 1979). Incorporation of label into hydrocarbons and pigments was found insignificant and has not been reported in any other helminth.

Among polar lipids, most of the label is found in components containing choline and ethanolamine as also in *H. diminuta* (Overturf and Dryer, 1968). In *H. contortus*, a greater part of the activity was found in phosphatidyl choline than in phosphatidyl ethanolamine, while the reverse was true for *H. diminuta*. However, the findings are in agreement with those obtained in *A. lumbricoides* (Jezyk, 1968), *S. mansonoides*, *S. mansoni* and *Dugesia dorotocephala* (Meyer et al., 1966*a*; 1966*b*). Both phosphatidyl choline and phosphatidyl ethanolamine are equally important constituents of membranes as these help in maintaining a continuity between the aqueous and lipid phases inside and outside of the cell, respectively. *H. contortus* resembles *H. diminuta* in that only trace amount of label is found in cerebrosides. However, in *S. mansoni* no label was found in cerebrosides. *H. contortus* also incorporated label from palmitic acid into phosphatidyl inositol and phosphatidyl serine as also reported in *S. mansoni*, *D. dorotocephala* and *S. mansonoides*. However, the biosynthesis of phosphatidyl serine from palmitic acid was insignificant. More synthesis of cerebrosides from HCO_3^- might be due to its fixation into succinate which is glycogenic.

It may be concluded that the adult *H. contortus* has extremely active mechanisms for the synthesis of complex lipids from exogenously supplied fatty acids and by CO_2 fixation. Further studies involving time-course incorporation of other precursors like glycerol may elucidate the precursor/product relationship.

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OESOPHAGOGASTRIC ULCER IN SWINE AND VITAMIN U
III. REDUCTION OF ECONOMIC LOSSES BY VITAMIN U

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Vitamin U (S-methylmethionine sulfonium salt) preparations imported from different sources were tested in large-scale pig fattening trials to determine their suitability for reducing the economic losses caused by oesophagogastric ulcer of pigs.

Five large-scale field trials were conducted; to the feed of 2639 pigs vitamin U was added regularly, while 3127 pigs were used as control.

By preventing aggravation of the ulcerative process, stimulating reparative ones and markedly reducing or completely preventing deaths due to haemorrhage, the treatment resulted in a 4.54% reduction of mortality and emergency-slaughtering, an 8.86% decrease of the specific feed utilization, a 7.52% increase of the average daily body mass gain, and a shortening of the mean duration of the fattening period by 13.3 days (the figures represent the mean, calculated from the five trials).

In the authors' opinion, economic losses caused by oesophagogastric ulcer can be considerably reduced if the pigs are fed vitamin U regularly, from the start of dry feeding up to the day of marketing for slaughter, in a quantity of 200 mg/kg feed.

Keywords. Swine, oesophagogastric ulcer, vitamin U.

In spite of the large number of publications dealing with factors considered to be the possible or actual causes of oesophagogastric ulcer of pigs (these publications were reviewed in detail by Kovács 1974; Kowalczyk, 1969; and O'Brien, 1969), the exact aetiology of the disease has remained unelucidated so far. Several authors presume that different factors can play a role in it separately or in cooperation. This hypothesis seems to be supported by the fact that no consistent success was achieved by prevention and treatment attempts aimed at supposed aetiological factors.

The unelucidated aetiology, the frequent incidence of the disease in Hungary (Kovács, 1974; Tamás and Bokori, 1979; Tamás et al., 1977*b*), and the considerable economic losses inflicted by it (Kovács, 1974; Tamás et al., 1977*a*, 1977*b*) prompted us to search for a compound suitable for reducing the economic losses caused by oesophagogastric ulcer, by preventing the disease from developing in large pig farms. Compounds which can be added to the mixed fodders without altering the given management and feeding technology are preferable for this purpose. Vitamin U (S-methylmethionine sulfonium salt), a compound with antihistamine and antiserotonine effect, was thought to meet all the above requirements. It has a general protective effect on epithelia, it stimulates the proliferation of epithelial cells, and has a therapeutic effect on gastric and duodenal ulcers of man (Hegedűs et al., 1977, 1983).

Materials and methods

Experimental design

Five large-scale pig fattening trials were conducted with different vitamin U preparations (a Merck preparation and a preparation obtained from the Soviet Union). All trials were conducted as follows: (*i*) the vitamin-fed (experimental) and the control groups were accommodated in the same building; (*ii*) the management and feeding, etc., technology was completely identical for both groups, and it did not differ from that used in the farm earlier; (*iii*) pigs of both groups received an industrially-processed mixed fodder of identical composition, showing no difference from that fed in the farm earlier, except that the feed of the experimental groups contained vitamin U; (*iv*) vitamin U, which had been mixed in the bran previously (vitamin U premix), was added to the feed when the industrial fodder mix was produced.

The lesions found in the stomachs of animals died or emergency-slaughtered during the experiments or slaughtered at the abattoir were classified as follows:

- no lesion was seen in the mucous membrane of the oesophageal part (pars oesophagica) of the stomach (code used in the tables and text: 1);
- mild, circumscribed lesions in the epithelium, restricted to the edge of mucosal folds (code: 2);
- severe, diffuse lesions in the epithelium (code: 3);
- epithelial lesions of varying severity, with one or more erosions (code: 4);
- characteristic small ulcer the size of a pinhead or cornel seed (code: 5);
- large, deep, characteristic ulcer (code: 6);
- ulcer healed with cicatrization (code: 7).

Trial I

The vitamin-treated group comprised 276 two months old pigs, and the untreated control group 515 animals of the same age. From the 60th day of life until the pigs reached the body mass of 105 kg, vitamin U (Merck) was fed continuously, in a quantity of 100 mg/kg of feed.

Trial II

The vitamin-treated group comprised 473 pigs. From the age of 10–12 days to 29 days the pigs were fed 50 mg/kg of feed, from 30 days of age 100 mg/kg of feed vitamin U (Merck), continuously. Feeding of vitamin U was terminated one month before the expectable date of slaughtering. The control group contained 580 pigs.

Trial III

The vitamin-treated group comprised 177 pigs. The feed was completed with 50 mg/kg of feed vitamin U (Merck) from 10–12 days of age, with 100 mg/kg vitamin U from 42 days of age, and with 150 mg/kg vitamin U from 80 days of age until the pigs reached the body mass of 105 kg. The control group comprised 184 pigs. This trial was conducted in a pig farm where oesophagogastric ulcer had caused severe economic losses during the previous years.

Trial IV

The vitamin-treated group comprised 1107 pigs. Pigs were fed 100 mg/kg of feed vitamin U (imported from the Soviet Union) from 10–12 days of age, 150 mg/kg vitamin U from 40 days of age, and 200 mg/kg vitamin U from 90 days of age until the pigs reached the body mass of 105 kg, continuously. The control group contained 1241 pigs.

Trial V

The vitamin-treated group comprised 606 pigs. The pigs were fed 200 mg/kg of feed vitamin U (imported from the Soviet Union) from 30 days of age up to the day of marketing for slaughter (i.e., the body mass of 105 kg), continuously. The control group comprised 607 pigs.

Results and evaluation

Trial I

1) In stomachs of pigs died or emergency-slaughtered during the experimental period (Table I)

(i) the incidence of lesions in animals lost from the control group exceeded 19.09%;

(ii) a considerable difference existed between the two groups in the severity of lesions. While in the vitamin-treated group the epithelial lesions (codes 2 and 3) predominated (18.18%), and large ulcers extending to deeper layers of the stomach wall (6) were absent, in the succumbed of emergency-slaughtered control pigs extensive ulceration (code: 6) was characteristic (36.36%);

(iii) in the control group, 15.4% of the total number of deaths was due to haemorrhage into the lumen of the stomach, starting from a large ulcer; no deaths due to such a cause occurred in the experimental group;

Table I

Results of stomach examination of pigs dropped out or presented at the abattoir, in %
(Trial I)

Code of lesions	Stomach examination results of			
	pigs dropped out		pigs presented at the abattoir	
	Experimental group	Control group	Experimental group	Control group
1	68.18	49.09	14.42	15.62
2	4.55	—	29.33	22.92
3	13.63	3.64	29.81	23.17
4	—	1.82	5.77	7.81
5	9.09	9.09	6.25	18.89
6	—	36.36	—	2.27
7	4.55	—	14.42	9.32

(iv) as opposed to the experimental group, in the control group no ulcer healed with cicatrization (7) was found.

2) The examination of stomachs of pigs presented at the abattoir (Table I) revealed that

(i) the disease showed nearly identical relative incidence in the two groups;

(ii) there were considerable differences between the two groups in the severity of lesions; namely, while in the experimental group mild lesions (2, 3 and 4) predominated (64.91%), in the control one severe lesions occurred in numerous pigs; no large ulcer (6) was found in the experimental group;

(iii) in the vitamin-treated group healing with cicatrization was considerably more frequent than in the control group. Of all the ulcerative lesions (5+6+7) 69.77 and 30.58% were ulcers healed with cicatrization (7) in the experimental and control group, respectively.

3) In the vitamin-treated group losses due to deaths and emergency-slaughtering were by 2.8% lower; the specific feed consumption was by 18.2% lower; the average daily body mass gain was by 6.7% higher; and the mean duration of the fattening period was by 13.3 days shorter than in the control group.

Trial II

1) In pigs died or emergency-slaughtered during the experimental period (Table II)

Table II

Results of stomach examination of pigs dropped out or presented as the abattoir, in %
Trial II

Code of lesions	Stomach examination results of pigs dropped out: Experimental group	pigs dropped out: Control group	pigs presented at the abattoir: Experimental group	pigs presented at the abattoir: Control group
1	69.09	40.74	3.49	3.08
2	7.27	6.48	9.01	20.93
3	10.90	11.11	65.11	42.73
4	3.64	8.33	2.62	7.05
5	1.82	10.19	3.78	12.34
6	3.64	20.37	—	2.64
7	3.64	2.78	15.99	11.23

(i) the incidence of lesions in the pars oesophagica of the stomachs of pigs dropped out from the control group was by 28.35% higher than the incidence in the experimental group;

(ii) while the experimental group was characterized by mild lesions (2, 3 and 4; showing an incidence rate of 21.81%), in the control group the occurrence of severe lesions (5 and 6) was typical (30.56%);

(iii) in the control group, death due to haemorrhage into the lumen of the stomach, starting from a large ulcer (6), occurred in 13 cases during the experimental period; these represented 20.0% of the total number of deaths and 59.1% (!!) of the pigs with large ulcers. In the vitamin-treated group no deaths due to haemorrhage occurred. In the control group a large proportion (81.1%) of the extensive ulcers (6) were found in the stomachs of pigs dropped out between 160 and 190 days of age. Also, 9 of the above-mentioned 13 deaths due to internal haemorrhage (69.2%) occurred in that period. It should be noted that in that period disturbances in drinking-water supply, lasting 2 ×3 days and extending to the whole farm, and a failure of feeding installations occurred, and fitting work of several days' duration inside the building was done at the same time;

(iv) in the experimental group, a high proportion (40%) of the ulcerative lesions healed with cicatrization (7); in the control group the corresponding percentage was 8.6%.

2) Before evaluating the results of stomach examinations performed at the abattoir (Table II), it should be mentioned that dosing of vitamin U was

terminated one month before the expected date of marketing for slaughter, in order to show whether it is justified to continue dosing of vitamin U up to the end of fattening.

In spite of terminating the administration of vitamin U before the end of fattening, it was found that

(i) in the vitamin-treated group mild and incipient lesions were preponderant;

(ii) characteristic ulcers (5 and 6) were found in 3.78 and 14.98% of the cases in the experimental and control group, respectively. At the same time, no large ulcers (6) were found in the experimental group;

(iii) in the experimental group the proportion of ulcers healed with cicatrization (7) among ulcerative lesions (5+6+7) was considerably higher (80.88%) than in the control group (42.86%).

3) In the experimental group, losses due to death or emergency-slaughtering were by 7.0% lower; specific feed consumption was 6.1% lower; the average daily body mass gain was by 9.8% higher; and the mean duration of the fattening period was by 18.6 days shorter than in the control group.

Trial III

1) The examination of stomachs of pigs dropped out (Table III) showed that

(i) the relative incidence of lesions was by 16.38% higher in the control group;

(ii) in the vitamin-treated group epithelial lesions (2 and 3; 37.92%),

Table III

Results of stomach examination of pigs dropped out or presented at the abattoir, in %
Trial III

Code of lesions	Stomach examination results of			
	pigs dropped out		pigs presented at the abattoir	
	Experimental group	Control group	Experimental group	Control group
1	41.38	25.00	0.72	—
2	17.24	7.14	14.18	1.50
3	20.68	7.14	47.52	27.61
4	6.90	14.29	6.38	10.45
5	6.90	14.29	11.35	25.37
6	6.90	32.14	5.67	31.34
7	—	—	14.18	3.73

while in the control group characteristic ulcerative lesions (5 and 6; 46.43%) were prevalent. The relative incidence of typical large ulcers (6) was 32.14% in the control, and 6.90% in the experimental group;

(iii) haemorrhage into the lumen of the stomach, starting from a large ulcer (6), was the direct cause of death in 27.3% and 3.7% of total deaths in the control and the experimental group, respectively;

(iv) cicatrized ulcers were not found in either of the groups.

2) According to the results of stomach examinations performed at the abattoir (Table III),

(i) oesophagogastric ulcer disease occurred in practically all the pigs in both groups;

(ii) while in the experimental group mild epithelial lesions (2 and 3) predominated (61.70%), in the control group typical ulcerative lesions (5 and 6) prevailed (56.71%). The relative incidence of large ulcers (6) was 31.34% in the control and 5.67% in the experimental group;

(iii) in the experimental group the proportion of cicatrized ulcers (7) among ulcerative lesions (5+6+7) was considerably higher (45.45%) than in the control group (6.17%).

3) In the experimental group, the losses due to deaths and emergency-slaughtering were by 6.8% lower; the specific feed consumption was by 8.7% lower; the average daily body mass gain was by 2.9% higher; and the mean duration of the fattening period was by 6.6 days shorter than in the control group.

Trial IV

1) The examination of stomachs of pigs dropped out due to death or emergency-slaughtering (Table IV) showed that

(i) the relative incidence of lesions in the stomach of pigs dropped out from the control group was by 17.16% higher than in the vitamin-fed group;

(ii) in the experimental group the epithelial lesions represented a considerably larger part of the cases than in the vitamin-fed group; the incidence of large ulcers (6) was 7.25% and 0.88% in the control and experimental group, respectively;

(iii) internal haemorrhage starting from a large ulcer (6) was the direct cause of death in 13.0 and 2.2% of the total fatal cases in the control and experimental group, respectively;

(iv) ulcer healed with cicatrization (7) was found in the stomach of 1 and 2 pigs in the experimental and control group, respectively.

Table IV

Results of stomach examination of pigs dropped out or presented at the abattoir, in %
Trial IV

Code of lesions	Stomach examination results of			
	pigs dropped out		pigs presented at the abattoir	
	Experimental group	Control group	Experimental group	Control group
1	86.73	69.57	0.28	—
2	6.19	8.70	11.78	5.05
3	2.66	5.07	57.73	35.19
4	0.88	2.16	7.80	11.19
5	1.78	5.80	8.23	14.19
6	0.88	7.25	7.80	32.61
7	0.88	1.45	6.38	1.77

2) The stomachs examined at the abattoir (Table IV) showed that

(i) oesophagogastric ulcer disease occurred in almost all of the pigs in both groups;

(ii) there were considerable differences in the severity of lesions; namely, while in the experimental group the epithelial lesions (2 and 3; 69.51%), in the control group the typical ulcers (5 and 6; 46.80%) were characteristic. The incidence of typical large ulcers (6) was 32.61% and 7.80% in the control and experimental group, respectively;

(iii) the proportion of cicatrized ulcers (7) within ulcerative lesions (5+6+7) was 28.48% in the experimental and 3.65% in the control group.

3) In the experimental group, losses due to deaths and emergency-slaughtering were by 4.5% lower; the specific feed consumption was by 5.6% lower; the average daily body mass gain was by 5.8% higher; and the mean duration of the fattening period was by 13.2 days shorter than in the control group.

Trial V

1) The examination of stomachs of pigs dropped out (Table V) indicated that

(i) the relative incidence of lesions was by 15.41% higher in the stomachs of control pigs;

(ii) in the experimental group the epithelial lesions (2 and 3; 48.84%), while in the control one the characteristic ulcerative

Table V

Results of stomach examination of pigs dropped out or presented at the abattoir, in %
Trial V

Code of lesions	Stomach examination results of			
	pigs dropped out		pigs presented at the abattoir	
	Experimental group	Control group	Experimental group	Control group
1	27.91	12.50	—	—
2	11.63	—	0.68	—
3	37.21	6.25	60.88	21.48
4	9.30	10.42	12.25	9.40
5	6.98	33.33	9.86	19.46
6	4.65	37.5	7.82	45.97
7	2.32	—	8.51	3.69

lesions (5 and 6; 70.83% !) were predominant. The incidence of large ulcers was 37.50% and 4.65% in the control and the experimental group, respectively;

(iii) internal haemorrhage starting from a large ulcer (6) was the direct cause of death in 11.1% and 5.9% of the total fatal cases in the control and experimental group, respectively;

(iv) no cicatrized ulcers were found in the control group.

2) The stomachs examined at the abattoir (Table V) revealed that

(i) the incidence of oeasophagogastric ulcer disease was 100% in both groups;

(ii) also in this trial, mild lesions (2 and 3) prevailed in the experimental group (61.56%), while in the control group the typical ulcerative lesions (5 and 6) were predominant (65.43%). Large ulcers occurred in 45.97% (! !) of the cases in the control group and in only 7.82% of the cases in the experimental group;

(iii) also in Trial V, the proportion of cicatrized ulcers (7) within ulcerative lesions (5+6+7) was considerably higher (32.47%) in the experimental group than in the control one (5.43%).

3) In the experimental group, losses due to deaths and emergency-slaughtering were by 1.6% lower; the specific feed consumption was by 5.7% lower; the average daily body mass gain was by 12.4% higher; and the mean duration of the fattening period was by 14.9 days shorter than in the control group, despite the fact that the control pigs were marketed at a lower body mass (101.30 kg) than the experimental ones (106.70 kg).

Discussion

1) The development of mucosal lesions in the stomach of fattening pigs could not be prevented by regular feeding of vitamin U in small doses. However, aggravation of the pathologic process was markedly inhibited, and the number of deaths due to severe ulcerative lesions (6) was considerably reduced. While in pigs dropped out or sent to the abattoir from the experimental groups mild epithelial lesions (2 and 3) predominated in all of the five trials, in the control groups usually the severe ulcerative lesions (5 and 6) prevailed. It is important to stress this finding from the following two aspects:

(i) The more severe the pathological process in a given animal is, the greater the *indirect losses* will be; indirect losses arise from reduced feed utilization, decreased average daily body mass gain, and prolonged fattening period.

(ii) Direct losses (deaths due to haemorrhage into the lumen of the stomach, or emergency-slaughtering in disease cases characterized by large ulcers (6) are neither negligible. Large differences were found between the experimental and control groups also in this respect. Namely, taking the mean of the five trials, in the groups fed vitamin U only 2.36%, while in the control groups 17.36% of the total number of deaths was due to internal haemorrhage. Losses are further aggravated by the fact that deaths due to haemorrhage usually occur in pigs of larger body mass (70–100 kg), in the last months of the fattening period.

2) In Trial II, the large number of deaths due to haemorrhage between 160 and 190 days of age supports the opinion of several authors (Barzoi et al., 1968; Curtin et al., 1963; Kovács, 1976; Dobos-Kovács et al., 1979; Reese et al., 1966; Tamás and Bokori, 1979; Tamás et al., 1978, 1983), namely, that stomach lesions induced by another cause or other causes can be considerably aggravated by adverse environmental factors (stressors) within a short time. Thus, in such cases the number of deaths due to haemorrhage into the lumen of the stomach, starting from a large ulcer (6), can show an abrupt increase.

By feeding vitamin U to the pigs the above-mentioned losses caused by adverse environmental factors can be prevented.

3) A certain part of the ulcerative lesions can undergo spontaneous healing (7) with cicatrization. However, in the experimental groups provided with vitamin U cicatrization of ulcers was considerably more frequent and more complete. E. g. in pigs presented at the abattoir, of the characteristic ulcerative lesions (5+6+7) only 51.4 and 17.7% were reparated ulcers (7) in the experimental and control groups, respectively (mean of the five trials). This suggests that vitamin U has an ulcer-healing effect. The existence of such an effect is supported by the fact that most of the few ulcers that developed in vitamin-treated animals failed to become extensive but quickly repaired instead, as indicated by the smaller, finer cicatrices found in the five experimental groups;

such cicatrices caused no deformation of the affected gastric area. Constriction of the oesophageal orifice due to cicatrization was observed very rarely. Contrarily, in the control groups the cicatrices were uneven and consistently accompanied by marked constriction of the oesophageal orifice.

Such oesophageal constrictions directly (mechanically) hinder feed intake and, indirectly, result in decreased average body mass gain, or even make emergency-slaughtering necessary.

4) The results of all field trials have proved that by adding vitamin U with preventive purpose to the industrial fodder mix fed in the usual way, without having to alter the management and feeding technology used in the given farm, it was possible to

(i) markedly reduce the losses due to deaths and emergency-slaughtering (mean of the five trials: by 4.54%);

(ii) improve the specific feed utilization (by 8.86% in the mean of the five trials);

(iii) increase the average daily body mass gain (mean of the five trials: by 7.52%);

(iv) shorten the mean duration of the fattening period (mean of the five trials: by 13.3 days).

5) From the evaluation of the results it has been concluded that the economic losses caused by the oesophagogastric ulcer of pigs can be reduced markedly if the pigs are continuously fed a diet supplemented with 200 mg/kg vitamin U from the start of dry feeding up to the day of marketing for slaughter.

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OESOPHAGOGASTRIC ULCER IN SWINE AND VITAMIN U
IV. EFFICIENCY OF THE HUNGARIAN VITAMIN U PREPARATION* IN REDUCTION OF ECONOMIC LOSSES

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A Hungarian vitamin U (S-methylmethionine sulfonium chloride) preparation was tested in large-scale pig fattening trials. The preparation was found to be as suitable for reducing economic losses due to oesophagogastric ulcer of pigs as the imported preparations used for this purpose earlier (the Merck preparation and the one obtained from the Soviet Union).

Two large-scale field trials were conducted, on a total of 528 experimental and 555 control animals. The feed of the former group was supplemented with vitamin U, 200 mg/kg of feed.

Vitamin U, by preventing aggravation of the pathologic process and deaths due to haemorrhage, by increasing regeneration (reparation), and as a result of other favourable effects, reduced the losses due to deaths and emergency-slaughtering by 4.2%, decreased the specific feed utilization by 19.2%, increased the average daily body mass gain by 10.5%, and shortened the mean duration of the fattening period by 9.2 days (mean of the two trials). To reduce the economic losses caused by oesophagogastric ulcer, the authors recommend to supplement the feed of pigs with 200 mg/kg of feed vitamin U from the start of dry feeding up to the day of marketing for slaughter.

Keywords. Pig, oesophagogastric ulcer, vitamin U.

In the accompanying paper (Tamás et al., 1986; see p. 81 in the present issue) we reported large-scale pig fattening trials in which two imported vitamin U preparations were tested for efficacy in reducing economic losses inflicted by oesophagogastric ulcer of swine. The results were favourable.

Recently, the REANAL Fine Chemicals Co. (Budapest) has elaborated a procedure for the production of vitamin U in Hungary. In the present study, the Hungarian vitamin U (S-methylmethionine sulfonium chloride) preparation was tested in two pig feeding trials, to determine whether it was as suitable for reducing the economic losses due to oesophagogastric ulcer as the imported preparations.

Materials and methods

Prior to testing in pig-feeding trials, the vitamin U preparation produced by REANAL was subjected to *laboratory analysis*. Its chemical composition and purity were tested by chromatography (on MN-300 cellulose-, Kieselgel-G and Fixion ion-exchange layers, in planar system), while its biological activity

* Produced by REANAL Fine Chemicals Co., Budapest, XIV. Telepes u, 53. Hungary. The experiment was conducted with financial support provided by REANAL Fine Chemicals Co.

was checked in microbiological model experiments (Hegedűs et al., 1977) in comparison with that of the import preparations.

The *pig-feeding trials* were conducted in an industrial-scale pig farm with an annual output of about 10,000 fattened pigs. In *Trial I*, the vitamin-fed (experimental) and the control group comprised 265 and 282 four weeks old pigs, respectively. In *Trial II*, the experimental group comprised 263, while the control one 273 four weeks old pigs. During the feeding trials, the management and feeding etc. technology was the same for both groups as that used in the farm earlier, and the experimental and control groups were always housed in the same building. The composition of industrial mixed fodders fed to the experimental and control pigs was always the same, with the only difference that to the feed of the former 200 mg/kg vitamin U preparation (produced by REANAL) was added and fed continuously from 28 days of age up to the day of marketing for slaughter.

The lesions found at the examination of stomachs of pigs died or emergency-slaughtered during the experiment and of those presented at the abattoir were classified as described in the accompanying paper (Tamás et al., 1986; see page 81 in this issue).

Results and evaluation

A) Results of laboratory analysis

1) The vitamin U preparation produced by REANAL showed a retention (R_f) value identical with that of the imported preparations marketed by the companies Merck, Fluka, Rudipont and Ms-Chemicals.

The level of methionine and homoserine contamination was at the detectability limit. No other ninhydrin-positive spots were seen.

2) The biological activity of the REANAL preparation was practically the same (Fig. 1) as that of the products marketed by the companies Merck, Rudipont and Ms-Chemicals. (The vitamin U preparation produced by Fluka is a bromide salt, while all the other preparations are chloride salts.)

B) Results of the pig-feeding trials

Trial I

1) The examination of stomachs of pigs spontaneously died during the experiment (Table I) showed that

(i) the relative incidence of lesions was by 14.3% higher in the control stomachs than in the stomachs of vitamin-fed pigs;

(ii) there was a marked difference between the two groups as regards the severity of lesions. While in the experimental group epithelial

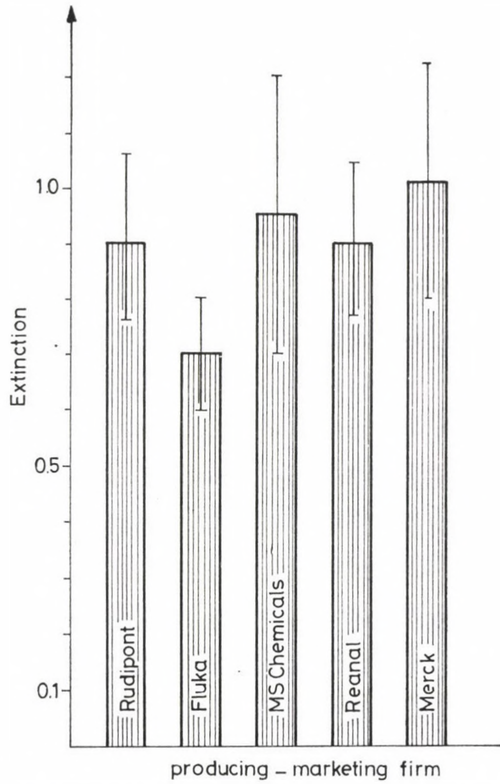

Fig. 1. Microbiological activity of various vitamin U preparations

Table I

Results of stomach examination of pigs died during the experiment, in %
Trial I

Lesions found in the pars oesophagica	Experimental group	Control group
No lesion in the mucous membrane	18.2	3.9
Mild, circumscribed epithelial lesion	—	—
Severe, diffuse epithelial lesion	45.5	19.2
Epithelial lesion of varying severity; with erosion	27.2	34.6
Small ulcer (the size of a cornel seed)	9.1	11.5
Large ulcer	—	30.8
Ulcer healed with cicatrization	—	—

lesions predominated (45.5%) and no large ulcers extending to the deeper layers of the stomach wall were found, in the control pigs died of oesophagogastric ulcer the typical ulcerative lesions (42.3%), and within them, formation of large ulcers, was typical (30.8%);

(iii) in the control group, death due to haemorrhage into the lumen of the stomach, starting from a large ulcer, occurred in 5 cases, representing 19.2% of total deaths and 62.5% of cases of large ulcer formation. No deaths due to this cause occurred in the experimental group;

(iv) no repaired (healed) ulcers were observed in either of the groups;

(v) in the stomach of one pig, died at 28 days of age (on day 0 of the experiment) already a severe epithelial lesion was found in the mucous membrane of the pars oesophagica. The first small ulcer, the size of a cornel seed, was observed in the stomach of a pig died at 40 days of age, while the first large ulcer in that of a control pig died at 45 days of age. In the latter animal, the direct cause of death was internal haemorrhage starting from the large ulcer.

2) According to results of stomach examinations performed at the abbattoir (Table II)

(i) all the pigs suffered from oesophagogastric ulcer disease in both groups;

(ii) in the experimental group ulcers were found in 36.6%, in the control group in 69.4% of the cases; large ulcers were observed in 13.3% and 34.7%, respectively;

Table II

Results of stomach examinations performed at the abattoir, in %
Trial I

Lesions found in the pars oesophagica	Experimental group	Control group
No lesion in the mucous membrane	—	—
Mild, circumscribed epithelial lesion	3.4	0.6
Severe, diffuse epithelial lesion	32.0	7.3
Epithelial lesion of varying severity, with erosion	9.3	14.7
Small ulcer (the size of a cornel seed)	23.3	34.7
Large ulcer	13.3	34.7
Ulcer healed with cicatrization	18.7	8.0

(iii) healing of the typical ulcers with cicatrization was considerably more frequent in the experimental group (33.7%) than in the control (10.3%).

3) Lesions in other organs:

(i) at autopsy of pigs died during the experiment it was found that enteritis and pneumonia were the causes of death in 18.2% and 57.7% of the cases in the experimental and control group, respectively;

(ii) at the examination of lungs at the abbatoir, milder or more severe, subacute or chronic bronchopneumonia or catarrhal-purulent pneumonia was found in the experimental and control groups with an incidence rate of 38.6% and 55.3%, respectively.

4) In the experimental group, losses due to death and emergency-slaughtering were by 5.3% lower, specific feed utilization was by 18.8% lower, average daily body mass gain was by 5.7% higher, and the mean duration of the fattening period was by 12.1 days shorter than in the control group.

Trial II

1) According to stomach examination results of pigs died during the experiment (Table III)

(i) the relative incidence of lesions in the pars oesophagica of the stomach was by 17.9% higher in the control group than in the experimental group;

(ii) in the experimental group the epithelial lesions (28.7%), while in the control group the typical ulcerative lesions (31.3%) predominated. The incidence rate of large ulcers was 7.1% and 25.0%, respectively;

Table III

Results of stomach examination of pigs died during the experiment, in %
Trial II

Lesions found in the pars oesophagica	Experimental group	Control group
No lesion in the mucous membrane	42.9	25.0
Mild, circumscribed epithelial lesion	—	—
Severe, diffuse epithelial lesion	28.7	18.7
Epithelial lesion of varying severity, with erosion	7.1	25.0
Small ulcer (the size of a cornel seed)	7.1	6.3
Large ulcer	7.1	25.0
Ulcer healed with cicatrization	7.1	—

(iii) in the control group, 3 deaths due to haemorrhage starting from large ulcers occurred, i.e. in 18.8% of the total number of deaths and 75.0% of pigs with large ulcers. No deaths due to this cause occurred in the experimental group;

(iv) no ulcers healed with cicatrization were found in the control group.

2) Stomach examinations performed at the abattoir (Table IV) indicate that

(i) in the control group all the animals were affected by the disease;

(ii) in the experimental group ulcerative lesions occurred in 36.3%, while in the control one in 69.5% of the cases. Large ulcers were found in 11.5% and 42.4% of the cases, respectively;

(iii) of ulcerative lesions, the proportion of ulcers healed with cicatrization was significantly higher in the experimental group (32.9%) than in the control (9.9%).

3) Lesions in other organs:

(i) enteritis and pneumonia acted as the cause of death in 14.3 and 43.8% of animals died in the experimental and the control group, respectively;

(ii) at the examination of lungs at the abattoir, more or less severe pneumonia was found in 37.3% and 57.9% of the experimental and control pigs, respectively.

4) In the experimental group, losses due to death and emergency-slaughtering were by 3.1% lower, the specific feed utilization was by 19.5% lower, the average daily body mass gain was by 15.3% higher, and the mean duration of the fattening period was by 6.2 days shorter than in the control group.

It should be noted that, due to circumstances independent of us, Trial II had to be terminated at the age of 224 days. Up to that age 59.9% of the ex-

Table IV

Results of stomach examinations performed at the abattoir, in %
Trial II

Lesions found in the pars oesophagica	Experimental group	Control group
No lesion in the mucous membrane	4.5	—
Mild, circumscribed epithelial lesion	5.1	—
Severe, diffuse epithelial lesion	27.4	8.5
Epithelial lesion of varying severity, with erosion	8.9	14.4
Small ulcer (the size of a cornel seed)	24.8	27.1
Large ulcer	11.5	42.4
Ulcer healed with cicatrization	17.8	7.6

perimental pigs and 46.5% of the control ones had been marketed for slaughter. Thus, the 6.2-day difference represents the difference between the above percentages of the experimental and control groups in the mean duration of the fattening period. The difference would have been greater if the stomachs had been examined later (at 105 kg body mass).

The growth rate of pigs in the control group was more uneven than that of the experimental pigs. At the termination of the experiment at 224 days of age the average individual body mass of the 86 pigs that remained in the experimental group was 91.5 kg, while that of the 126 pigs that remained in the control group was only 72.4 kg.

Discussion

Feeding trials using a preventive dose (200 mg/kg of feed) of the Hungarian vitamin U preparation gave similar results as our previous experiments (Tamás et al., 1986; see the accompanying paper, p. 81 in this issue) in which imported vitamin U preparations were used.

1) Namely, at the examination of stomachs of pigs died during the experiment and of those presented at the abbatoir it was found that

(i) the development of severe forms of oesophagogastric ulcer was prevented;

(ii) healing of typical ulcers was stimulated;

(iii) deaths due to haemorrhage into the lumen of the stomach, starting from large ulcers, could be prevented. Namely, in the control group 19.0% of total losses (mean of the two experiments) was due to haemorrhage into the lumen of the stomach, while no deaths due to such a cause occurred in the experimental groups.

2) There were differences between the experimental and control groups also in the lesions found in other organs (lungs, intestine) of pigs died during the experiment and of those presented at the abattoir, since (in the mean of the two experiments)

(i) in animals died during the experiments the relative incidence of pneumonia and enteritis as the cause of death was by 34.5% lower in the experimental groups;

(ii) in pigs presented at the abattoir the incidence of pneumonia was by 18.7% lower in the experimental groups than in the control groups.

3) Due to the above-listed advantageous effects of vitamin U, its preventive dose (200 mg/kg of feed) resulted in markedly improved fattening results; namely, the losses due to deaths and emergency-slaughtering were reduced by 4.2%, the specific feed utilization was improved by 19.2%, the average daily

body mass gain increased by 10.5%, and the mean duration of the fattening period was shortened by 9.2 days.

4) It was also in Trial I that the mild form of oesophagogastric ulcer was already present in the stomach of a pig died at 28 days of age; furthermore, in a 45 days old pig haemorrhage starting from a large ulcer was the cause of death. Since earlier we had observed that oesophagogastric ulcer occurred already in few days old piglets (Tamás et al., 1978), furthermore, death due to haemorrhage occurred already in 34 days old (Tamás et al., 1978) or even in 29 days old (Tamás et al., 1981) piglets, we recommend to provide piglets with vitamin U from the start of dry feeding up to the day of marketing for slaughter, regularly.

5) Similarly to the preventive dose of the imported vitamin U preparations, the preventive dose of the Hungarian vitamin U preparation tested by us also failed to prevent the development of epithelial lesions in the pars oesophagica of the stomach. This is not surprising since a 100% "anti-ulcer" and "healing" effect of vitamin U manifests itself only at a much higher dose (about 0.1 g/kg body mass daily), even in the case of experimentally induced peptic ulcers (Hegedűs et al., 1980). The low preventive vitamin U dose administered by us corresponds to only 0.01 g/kg body mass/day even at the beginning of the piglet-rearing period. Thereafter the daily dose calculated for body mass decreases further, and e.g. for pigs of 80 kg body mass it is only about 0.007–0.008 g/kg body mass. It is only a question of economical character to determine the level to which the vitamin U dose should be increased to achieve maximum profitability in pig production. Since fattening results can be improved considerably and, at the same time, economically already by using the 200 mg/kg of feed preventive dose proposed by us, this concentration is recommended for use in the practice also in the future.

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LYMPHOSARCOMA IN PIKE-PERCH (*STIZOSTEDION LUCIOPERCA* L.): A CASE REPORT

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A tumour the size of a nut was observed on a pike-perch of about 2000 g body mass, caught in the upper reaches of the River Tisza. By histological examination the neoplasm proved to be a lymphosarcoma. Although the foreign literature is abundant in data on neoplasms of the pike-perch, in Hungary no tumours have been reported in this fish species so far.

Keywords. Lymphosarcoma, pike-perch (*Stizostedion lucioperca* L.), histopathology.

During the regular pathological examination of fish, neoplastic growths and lesions are observed rather infrequently, since, as opposed to other diseases, neoplasms are usually of sporadic occurrence. Sporadic neoplasms may escape the attention of the non-professional examiner because they are rarely associated with apparent disease or deaths. In spite of this fact, various neoplastic diseases are being reported in increasing number in several species of fish, both in salt- and freshwater fishes. Schlumberger and Lucké (1948) gave a good account of these lesions, commented upon by Mawdesley-Thomas (1975) from the morphological and aetiological point of view. Descriptions and reports have been published at an increased rate since then, particularly on lesions occurring in large numbers within a circumscribed geographic area (Ljumberg, 1976; Sonstegard, 1975) and on neoplasms from which viruses can be isolated, or demonstrated by electron microscopy.

Nowadays, research activities are focussed on the aetiology of neoplastic diseases. Although viruses have been proved to have a role in the aetiology of several neoplastic diseases, e.g. the stomato-papillomatosis of eels (McAllister, 1977), in other disease cases no infectious agents could be demonstrated. Primarily in external lesions occurring on the body surface are environmental pollution, various biocides, polychlorinated diphenyls and detergents suspected to have a role (Mawdesley-Thomas, 1975).

The pike-perch of about 2000 g body mass originated from the catch of a fishery co-operative of the Upper Tisza region. The pathologically altered part was excised and fixed in 10% neutral formalin. Of the fixed material, paraffin-embedded sections were cut, and stained with haematoxylin and eosin.

On the tail stem of the pike-perch, ventrally, near the anus and the anal fin, a scaleless protrusion the size of a nut, compact to the touch and having

a tuberous surface was observed. The outer layers of the affected area were necrotic, with signs of haemorrhage here and there (Fig. 1). In cross-sections of the altered region it was apparent that the disease process had extended into the muscle layer. At dissection, no pathological lesions were seen in the internal organs.

Light microscopy revealed that the altered areas were devoid of epithelium, and the corium and subcutis were infiltrated by tumour cells (Fig. 2). In the dense connective tissue layer of the corium the collagen fibres had become pushed apart and widened to several times of their original size (Fig. 3). The interfibrillar spaces were filled with masses of neoplastic cells having an intensely staining nucleus and little cytoplasm. The loose connective tissue layer of the subcutis was also widened and contained similar cells in foci or dispersed. In the muscular layer, tumour cell infiltration progressed in the myosepta separating the muscle bundles. Here and there the tumour cells penetrated the interfibrillar spaces as well. In the severely infiltrated areas, primarily in the corium and less extensively in the muscular layer, cell degeneration and necrosis occurred both in the infiltrating and in the somatic cells (Fig. 4).

No signs indicative of inflammation or demarcation, i.e. host response, could be observed.

Based upon the histopathological findings, the mass proved to be a lymphosarcoma of lymphocytic character.

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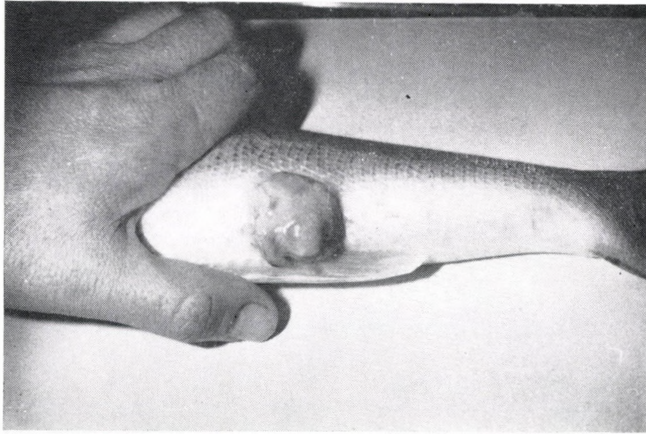

Fig. 1. Lymphosarcoma appearing as a tuberous mass the size of a nut.

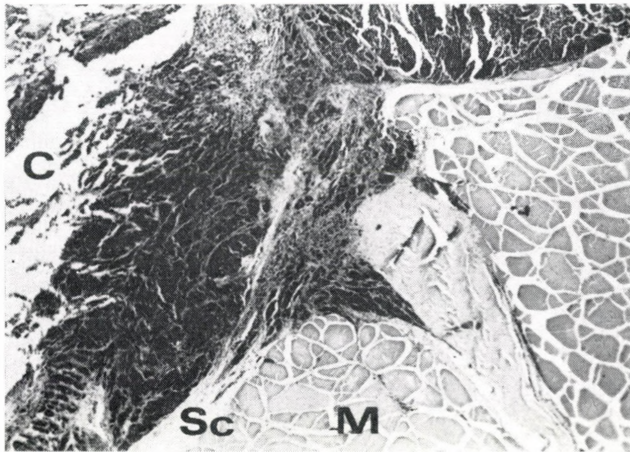

Fig. 2. Infiltration of the corium (C) and subcutis (Sc) by tumour cells; M = muscular tissue. H–E., appr. $\times 200$

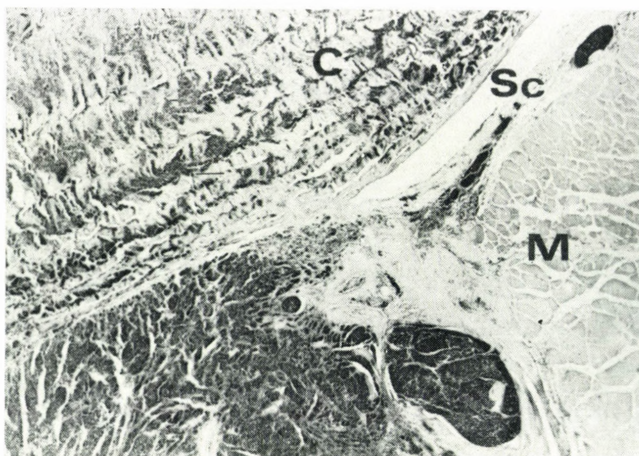

Fig. 3. In the dense connective tissue layer of the corium (C) the collagen fibres have become pushed apart and the spaces are filled with lymphocyte-like cells. H–E., appr. ×200

Fig. 4. Infiltrative growth of the tumour between muscle fibres and in the myoseptum. H–E., appr. ×200

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BLOOD GAS AND ACID-BASE VALUES IN DOGS WITH EXPERIMENTALLY INDUCED HYPOPOTASSAEMIA

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Experimental hypopotassaemia (hypokaliaemia) was induced in 11 mongrel dogs by treatment with a cation exchanger (Resonium-A®) and a diuretic (Hypothiazide®) on 5 successive days. Venous blood samples were withdrawn daily and analysed for serum, whole-blood and intracellular K^+ levels, serum Na^+, blood gas and acid-base values.

The experimentally-induced decrease in the extracellular K^+ concentration accounted for a shift of the acid-base balance towards metabolic alkalosis. The changes in mean base excess (BE), extracellular base excess (EBE) and actual bicarbonate (HCO_3^-) were highly significant, whereas the changes in blood pH were of low significance during the hypopotassaemic condition. No appreciable changes were observed in the mean blood gas (pCO_2, pO_2), O_2-saturation and haemoglobin values.

Keywords. Hypopotassaemia, dog, blood gas, acid-base balance, serum K^+, Na^+, haemoglobin.

Electrolyte and acid-base disturbances are common in carnivores, and usually associated with diseases of the digestive tract, chronic renal disease, circulation disease. Supervening complications as a rule aggravate the primary disease, not infrequently to the degree of life hazard. Cornelius and Rawlings (1981) demonstrated acid-base disturbances in 28% of 220 dogs diseased with various symptoms, such as vomiting, diarrhoea, dehydration, polydipsia, polyuria and dyspnoea. Sixty-nine per cent of the dogs showed metabolic acidosis, 13% respiratory acidosis, 15% metabolic alkalosis, and 3% respiratory alkalosis.

Muir (1982) investigated the acid-base and electrolyte status of 57 dogs with gastric dilatation volvulus, and demonstrated acid-base disturbances in 38; 32 suffered from metabolic acidosis, which was associated with hypopotassaemia in 11 (34.3%); metabolic or respiratory alkalosis was diagnosed in 6 dogs; the latter condition was associated with hypopotassaemia in five cases (83.3%).

Against this, Wingfield et al. (1982) found normal acid-base, K and Na values in all of 20 dogs with gastric dilatation volvulus.

Although the electrolyte and acid-base status of dogs is of great practical importance, as far as we are informed only short-term experimental hyper- or hypopotassaemia has been studied for influence on these parameters (Coulter

and Engen, 1972; Coulter et al., 1975). To obtain more information on the problem, we examined the changes taking place in the blood gas and acid-base values of dogs during experimental hypopotassaemia lasting several days.

Materials and methods

Eleven clinically healthy mongrel dogs of both sexes, 2 to 4 years old, and 8 to 17 kg in body mass, were used.

The initial blood gas and acid-base values were determined on the first day of the experiment. Hypopotassaemia was induced by oral administration of Na-polystyrene-sulphonate (Resonium-A®, Winthrop Ltd., England) in aqueous solution through an oesophageal tube, twice daily at the dose level of 1 g per kg body mass, and with hydrochlorothiazide (Hypothiazide®, Chinoin, Hungary) daily, in a dose of 10 mg per kg body mass. The hypopotassaemic condition was maintained by this treatment for 5 successive days. During the experiment the dogs were caged singly and received a commercial dog food (Kuke delikát®, Agricultural Cooperative, Tahitótfalu, Hungary) and drinking water ad libitum.

Further to regular clinical observation throughout the period of study, blood samples were withdrawn from the jugular vein of the dogs daily (at 9.00 a.m.), for determination of the K^+, Na^+, blood gas, acid-base, haemoglobin and haematocrit values.

Whole blood and serum K^+, and serum Na^+, were determined by flame photometry (Pulfrich photometer, FRG). Intracellular K^+ was calculated from whole blood and serum K^+ and the haematocrit value. Heparinized blood samples were withdrawn under anaerobic conditions and stored in melting ice until used in pH, pCO_2, and pO_2 assays, which were carried out in an ABL 2 acid-base analyser (Radiometer, Copenhagen, Denmark) at 37 °C, within half an hour after withdrawal. The pH, pCO_2 and pO_2 values were corrected for 39 °C (Siggaard-Andersen, 1963), the average body temperature of the dog. The other parameters required for determination of the acid-base value, such as base excess (BE), extracellular base excess (EBE), and actual bicarbonate (HCO_3^-), were calculated from the pH, pCO_2 and haemoglobin (Hb) values.

The Hb concentration was determined with the cyanmethaemoglobin method, the haematocrit (HT) value with an Erythrovolmet (Fok-Gyem, Hungary) apparatus. Oxygen saturation (SAT) was calculated according to Marsoner and Harnoncourt (1976).

The mean values of all the above parameters were calculated for every day, and were compared to the initial value by one-way analysis of variance (Steel and Torrie, 1980).

Results

The dogs tended to become increasingly apathic and less resistant to interventions with the progression of experimental hypopotassaemia. They showed a slight movement incoordination, staggered, rose and moved reluctantly. Flaccidity and occasional convulsive contractions of extremity muscles were observed in recumbency. Inappetence, nausea, bloating and constipation were developed in succession. The water intake was only slightly increased, but a distinct polyuria was developed during the five days. Slowly progressive bradycardia turned severe by the end of the experimental hypopotassaemia.

The initial mean serum K^+ level, 4.31 mmol/l, decreased gradually to 3.07 mmol/l by the fifth day. Difference from the initial value was significant throughout, at $P < 0.01$ on the second day, and at $P < 0.001$ on all further days.

The mean K^+ level tended to decrease slightly in the whole blood, but did not change intracellularly during the period of study.

The mean serum Na^+ decreased from 158.2 mmol/l to 150.3 mmol/l by the end of experimental hypopotassaemia; the difference from the baseline was significant at the $P < 0.01$ level on days 3 and 5 (Table I).

The mean BE and EBE values changed from —1.87 mmol/l to +2.87 mmol/l and from —1.5 mmol/l to +3.65 mmol/l, respectively, during the period of study. The increase over the baseline was highly significant ($P < < 0.001$) from the second day of the experiment on.

Table I

Mean values of K^+, Na^+, haematocrit and heart rate (HR) during the 5-day hypopotassaemic condition

Days	K^+ (mmol/l)			Na^+ (mmol/l)	Haematocrit value	HR (beats/min)
	serum	whole blood	intracellular			
1	4.31	6.21	8.23	158.2	0.49	157.0
2	3.72	6.07	8.28	154.1	0.52	143.6
3	3.44	5.98	8.26	151.3	0.53	129.6
4	3.19	5.76	8.29	154.0	0.52	113.7
5	3.07	5.69	8.17	150.3	0.53	106.1
F value*	12.38***	0.80	0.01	3.83*	0.64	6.95***
P = 0.05	0.40	0.68	1.21	4.74	0.07	22.3
P = 0.01	0.52	0.88	1.62	6.16	0.09	29.0
P = 0.001	0.69	1.17	2.11	8.19	0.13	38.7

* $P < 0.05$; *** $P < 0.001$

The mean HCO_3^- value rose from 23.71 to 28.65 mmol/l. The increase over the baseline was highly significant, at $P < 0.01$ on days 2 and 3, and at $P < 0.001$ on days 4 and 5.

The blood pH rose slightly during experimental hypopotassaemia. The significance of difference from the baseline was at the $P < 0.05$ level on all days except day 3, when it was higher ($P < 0.01$).

The mean pCO_2, pO_2, Hb and SAT values did not appreciably change during the hypopotassaemic condition (Table II).

The mean haematocrit value showed a slight, not significant, increase throughout.

The initial heart rate, 157.0 beats/min decreased to 106.1 beats/min by the fifth day; the difference was highly significant, $P < 0.001$, on days 4 and 5.

Discussion

Minor changes in the extracellular K^+ concentration cause disturbances in intracellular metabolism, above all of carbohydrates. Together with Ca^{++}, extracellular K^+ plays an important role in the normal function of the heart and muscles (Ettinger and Suter, 1970; Parker and Adam, 1977; Rubin, 1968). In physiological conditions intra- and extracellular K^+ levels are in equilibrium, which may, however, change in either direction under certain influences.

The cation-exchanger and diuretic treatment applied caused a significant decrease in the extracellular level of K^+, but had no influence on its intracellular level. Extracellular hypopotassaemia accounted for a characteristic weakness of the striated muscles, and for movement incoordination,

Table II

Values of the acid-base parameters on the 5 days of experimental hypopotassaemia

Days	pH	PCO_2 kPa	PO_2 kPa	Hb mmol/l	BE mmol/l	EBE mmol/l	HCO_3^- mmol/l	SAT %
1	7.313	6.50	5.23	10.36	−1.87	−1.50	23.71	59.63
2	7.356	6.66	4.92	10.59	1.48	1.93	26.76	58.95
3	7.370	6.72	4.53	10.73	2.46	3.02	27.64	61.00
4	7.357	7.12	5.19	10.27	2.93	3.70	28.66	61.93
5	7.354	7.28	5.39	10.06	2.87	3.65	28.65	64.34
F value*	3.12*	1.18	0.76	0.34	10.02***	8.78***	6.27***	0.26
P = 0.05	0.034	0.96	1.09	1.29	1.80	1.97	2.31	11.84
P = 0.01	0.045	1.25	1.41	1.68	2.34	2.56	3.00	15.39
P = 0.001	0.059	1.66	1.88	2.23	3.11	3.40	3.99	20.48

* $P < 0.05$; *** $P < 0.001$

listlessness, and decreased muscle function. Smooth muscle dysfunction was most conspicuous in the gastrointestinal tract, to judge from loss of appetite, occasional nausea, decrease in intestinal peristalsis and, finally, constipation. A significant decrease in the heart rate was also observed. Decrease in extracellular K^+ was associated with a slight decrease in Na^+, in consequence of increased diuresis.

For evaluation of the acid-base parameters determined, we relied for reference on those assessed by Muir (1982) in venous (jugular) blood samples of 100 clinically healthy dogs as follows: pH 7.36 (7.25–7.50); pCO_2 34.9 (26–42) mmHg; pO_2 36.6 (33–40) mmHg (mmHg $\times$ 0.133 = kPa); plasma bicarbonate 21.6 (15–25) mmol/l; BE —3.0 (—5.7–+1.2) mmol/l.

The initial blood pH and pCO_2 determined by us was somewhat lower and higher, respectively, probably because of correction of the measured values for 39 °C body temperature (Siggaard-Andersen, 1963); in Muir's cited report, information is lacking about the temperature at which the measurements were performed.

The five-day experimental hypopotassaemia, induced with cation exchanger and diuretic treatment, accounted for a gradual decrease in the extracellular K^+ concentration, and thereby for a shift of the acid-base status towards metabolic alkalosis, to judge from a statistically significant increase in the mean BE, EBE and HCO_3^- values, while the pH of the venous blood rose only slightly.

According to Coulter and Engen (1972), hyperpotassaemia (8.3 $\pm$ 2.3 mmol/l), induced by parenteral administration of isotonic KCl (11.2 g KCl in one litre of anion-free H_2O) was associated with a parallel decrease in blood pH from 7.27 $\pm$ 0.07 to 7.22 $\pm$ 0.06, without, however, any alteration in blood pCO_2 and pO_2, exactly as in our study.

It appears that changes in the extracellular K^+ concentration cause an opposite shift in the acid-base balance, so that hypopotassaemia may ultimately lead to metabolic alkalosis, whereas hyperpotassaemia (Coulter and Engen, 1972) to metabolic acidosis.

Acknowledgements

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ACID-BASE BALANCE OF DOGS IN OBSTETRICAL CLINICAL PRACTICE

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The acid-base status of 63 dogs operated with different obstetrical indications was investigated. Attention is called to the fact that the actual acid-base status of dogs can be best evaluated on the basis of the base excess (BE) value. In accordance with data of the literature, BE values between +2.5 and —5.9 mmol/l can be considered physiological in clinically healthy dogs.

Of the 46 dogs operated because of pyometra, cystic glandular hyperplasia (hyperplasia glandularis cystica; HGC) or dystocia (partus gravis), 27 (58.7%) had physiological base excess (BE) values, while 19 (41.3%) showed an expressed metabolic acidosis (with BE values lower than —6 mmol/l). Of the dogs having BE values lower than —9 mmol/l, 3 died within 24 h after the operation (on day 0 or 1).

Attention is called to the fact that in our days measurement of the actual acid-base status is indispensable before performing obstetrical operations on clinically diseased animals.

Keywords. Acid-base status, base excess (BE), dog, obstetrics, operation.

The actual acid-base status of dogs can be determined from the arterial, capillary and venous blood as well. The arterial acid-base values considered physiological for clinically healthy dogs by various authors are shown in Table I.

Under practical conditions arterial blood sampling is difficult to perform particularly from non-anaesthetized dogs of small body size. At the same time the stress constituted by unfamiliar stimuli (securing the animal, bandaging its mouth, laying it down for blood sampling, etc.) may elicit resistance accompanied by hypo- or hyperventilation which might lead to a shift of the acid-base balance (Rodkey et al., 1978). According to the studies of Rodkey et al. (1978) and Sandmann et al. (1983), the acid-base balance of the capillary blood withdrawn from the marginal parts of the ear or footpad is nearly identical with that of the arterial blood (Table II). An advantage of capillary blood sampling is that it is easy to perform on non-anaesthetized patients of smaller body size as well. On the other hand, several authors (Rodkey et al., 1978; Sluijs et al., 1983) call attention to the fact that in hypovolemic shock capilperipheral circulatory disturbances the acid-base values measured in the and lary blood cannot be replaced by those of the arterial blood.

As compared to the arterial blood, the pH and pCO_2 of the venous blood is slightly lower and higher, respectively, while its pO_2 value is much lower (Table II). Other authors (Carter and Brobst, 1969; Bright and Green, 1976),

Table I

Mean values of parameters characterizing the acid-base balance, measured in the arterial blood of clinically healthy dogs

Authors	pH	pCO_2 mmHg*	pO_2 mmHg*	BE mmol/l	HCO_3^- mmol/l
Rodkey et al. (1978)	7.416	33.9	82.7	−1.9	21.2
Rose and Carter (1980)	7.372	32.5	96.2	−5.0	—
Cornelius and Rawlings (1981)	7.450	31.0	90.7	—	20.9
Wingfield et al. (1982)	7.386	31.6	79.7	—	18.2
Sandmann et al. (1983)	7.416	36.1	97.6	+0.2	22.7

* 1 mmHg × 0.133 = kPa

however, reported a larger pH difference (0.03–0.10) between arterial and venous blood. As opposed to pH, there is no appreciable difference in the metabolic parameters, i.e. base excess (BE) and actual bicarbonate (HCO_3^-), as shown in Table II. Thus, the quantity of buffer solution needed to compensate for acidosis can be assessed with high probability also from the values measured in the venous blood.

The aim of the present investigations was to assess how frequently disorders of acid-base balance should be reckoned with in the clinical practice of small-animal obstetrics, in cases with various operative indications.

Materials and methods

The acid-base status of 63 female dogs of different breeds and age was determined from the venous blood withdrawn immediately prior to anaesthesia for operation.

The number of operations performed because of mammary tumour (neoplasma mammae), dystocia (partus gravis), cystic glandular hyperplasia

Table II

Differences between the mean acid-base values of arterial and capillary, and between those of arterial and venous blood

Parameter	Arterial ⟷ Capillary blood		Arterial ⟷ Venous blood	
	A	B	A	B
pH	0.016	−0.004	− 0.011	−0.046
pCO_2, mmHg	−0.5	0.7	2.7	8.3
pO_2, mmHg	1.2	−5.6	−30.6	52.1
BE, mmol/l	−0.7	0.2	− 0.6	0.6
HCO_3^-, mmol/l	−0.6	0.2	1.1	−2.4

A: Rodkey et al. (1978); B: Sandmann et al. (1983)

(hyperplasia glandularis cystica; HGC), pyometra, or with the purpose of ovariectomy, is shown in Table III. Blood samples were withdrawn from the vena saphena parva under anaerobic conditions into heparinized syringes. The syringes were immediately closed airtight with a rubber cap, and stored in melting ice until used for the measurements. The samples were assayed for pH, pCO_2 and pO_2 with an acid–base analyser (ABL 2, Radiometer, Copenhagen, Denmark) within half an hour, at 37 °C. The obtained values were corrected for 39 °C (Siggaard-Andersen, 1963), the average body temperature of the dog. The haemoglobin (Hb) concentration was determined by the cyanmethaemoglobin method. The other parameters characterizing the acid-base status [BE, extracellular base excess (EBE), HCO_3^-] were calculated by using the equation constructed for human blood (Szenci and Nyírő, 1981), since the total protein and haemoglobin concentrations of canine blood are practically identical with those of human blood (Shalm et al., 1975). From the obtained values statistical means ($\bar{x} \pm SD$) were calculated and compared to one another by one-way, independent-sample analysis of variance (Steel and Torrie, 1980).

Results

The results of the acid-base measurements performed before the obstetrical operations of various indications are given in Table III.

No appreciable difference was found between the pH means of the different groups. Animals to be operated with the aim of ovariectomy and those with mammary tumour had higher pCO_2 means than those operated because of partus gravis, HGC and pyometra. At the same time, the latter two groups had significantly lower pO_2 means. The mean haemoglobin concentrations of clinically diseased animals were lower than those found for healthy dogs; in dogs operated with pyometra and dystocia the difference reached the level of significance.

The metabolic parameters (BE, EBE, HCO_3^-) showed a shift towards expressed metabolic acidosis in the following order of severity: dogs with pyometra, HGC, and dystocia.

Table IV was constructed on the basis of BE values measured in the blood withdrawn prior to operations of different indications. The blood BE value of clinically healthy dogs (ovariectomy, mammary tumour) was between +2.5 and —3.5 mmol/l in 64.7% of the cases, while in the remaining dogs it was between —3.6 and —5.9 mmol/l. In 10 (21.7%) of the 46 clinically diseased animals the BE value ranged between —6 and —9 mmol/l, whereas in 9 (19.6%) a value lower than —9 mmol/l was measured. Three dogs of this latter group died soon after operation (on days 0 or 1). Of the remaining dogs,

Table III

Monitoring the acid-base balance of dogs

Parameter / Group	a	b
n	12	5
pH	7.287 ± 0.056	7.270 ± 0.019
pCO_2 (kPa)	7.0 ± 1,2	7.0 ± 0.6
pO_2 (kPa)	5.3 ± 0.8^{b**}	7.1 ± 1.8
Hb (mmol/l)	11.0 ± 1.5	10.3 ± 0.9
BE (mmol/l)	−2.4 ± 1.8	−3.1 ± 2.2
EBE (mmol/l)	−1.6 ± 1.9	−2.3 ± 1.2
HCO_3^- (mmol/l)	23.9 ± 2.0	23.4 ± 2.6
Age (years)	2.6 ± 2.4	6.0 ± 2.9

a: ovariectomy; b: neoplasma mammae (mammary tumour); c: hyperplasia glandularis cystica (HGC); d: pyometra; e: partus gravis (dystocia)

58.7% had BE values practically identical with those of the clinically healthy animals.

Discussion

Muir (1982) measured the parameters characterizing the acid-base status of clinically healthy dogs (n = 100) in blood samples withdrawn from the jugular vein. He reported the following values: pH = 7.36 (7.25–7.50); pCO_2 =

Table IV

Base excess (BE) values as determined before obstetrical operations of different indications

Type of operation	BE, mmol/l				
	+2.5–0	0—−3.5	−3.6—−5.9	−6—−9	<−9
Ovariectomy	1	8	3	—	—
Neoplasma mammae	0	2	3	—	—
Total	1 (5.9%)	10 (58.8%)	6 (35.3%)		
Hyperplasia glandularis cystica	—	2	2	2	1
Pyometra	3	5	12	7	6*
Partus gravis	—	1	2	1	2
Total	3 (6.5%)	8 (17.4%)	16 (34.8%)	10 (21.7%)	9 (19.6%)

* Of them, 3 dogs died on day 0 or 1 post-operation

prior to different obstetrical operations

c	d	e
7	33	6
7.275 ± 0.038	7.278 ± 0.070	7.290 ± 0.044
5.9 ± 1.1 [a, b*]	6.1 ± 1.4	5.2 ± 0.5 [a, b**]
4.6 ± 1.0 [b***, e*]	4.2 ± 1.6 [b***, e*]	6.1 ± 1.4
9.5 ± 2.0	8.5 ± 2.0 [a**, b*]	8.7 ± 1.8 [a**]
−5.6 ± 4.0 [a*]	−5.2 ± 3.4 [a*]	−6.7 ± 2.8 [a**, b*]
−5.4 ± 4.3 [a**, b*]	−5.0 ± 3.5 [a, b*]	−6.8 ± 2.6 [a, b***]
20.0 ± 4.4 [a, b*]	20.3 ± 3.3 [a, b*]	18.2 ± 2.4 [a, b*]
9.7 ± 3.4	8.1 ± 2.8	6.1 ± 3.4

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

34.9 (26–42) mmHg (mmHg×0.133 = kPa); pO_2 = 36.6 (33–40) mmHg; BE = —3.0 (—5.7–+1.2) mmol/l; HCO_3^- = 21.6 (15–25) mmol/l. On the other hand, Rodkey et al. (1978) and Sandmann et al. (1983) found the mean pH, pCO_2, pO_2 and HCO_3^- values of blood samples withdrawn from the vena cephalica antebrachii slightly higher, while the BE value somewhat lower. In the clinically healthy dogs of our test material, the mean pH, pO_2 and pCO_2 values of the blood were found to be slightly lower and higher, respectively. At the same time, there was no appreciable difference in the metabolic parameters.

The differences obtained between the mean values, which hold true also for values measured in arterial blood (Table I), can presumably be traced back to the fact that pH, pCO_2 and pO_2 were measured at different temperatures [Rodkey et al. (1978) at 38 °C] or were corrected for the actual body temperature (Cornelius and Rawlings, 1981; Wingfield et al., 1982), while other authors failed to specify the temperature range in which their measurements had been carried out (Rose and Carter, 1980; Muir, 1982; Sandmann et al., 1983; Sluijs et al., 1983). Since the prevailing pH, pCO_2 and pO_2 values depend on the body temperature, correction of the values measured at 37 °C for the actual or average body temperature is indispensable (Siggaard-Andersen, 1963).

At the same time it must not be disregarded that unusual environmental stimuli may evoke resistance from the animals' part and induce hypo- or hyperventilation which, depending on their degree, might lead to a shift of the acid-base balance towards respiratory acidosis (Rodkey et al., 1978). In our test material the higher pCO_2 values of clinically healthy dogs as compared to the other groups support this statement.

Since in a considerable proportion of cases the metabolic parameters (BE, EBE) are scarcely, or not at all, influenced by the environmental factors, these parameters reflect the actual acid-base status in a reliable manner. In accordance with the results of Muir (1982), the BE values obtained for clinically healthy dogs (between +2.5 and —5.9 mmol/l) can be considered physiological.

Sixty % of the dogs operated with pyometra and HGC had physiological BE values, whereas in the remaining 40% there was an expressed metabolic acidosis (BE < —6 mmol/l). Three of the dogs having BE values lower than —9 mmol/l died soon after the operation (on day 0 or 1). Our results are consistent with those of Cornelius and Rawlings (1981), who observed acid-base disturbances (metabolic acidosis in 69, respiratory acidosis in 13, metabolic alkalosis in 15, and respiratory alkalosis in 3% of the cases) in 28% of the dogs exhibiting various clinical symptoms (vomiting, polydipsia, polyuria, dehydration). The significance of this finding is further emphasized by the fact that in patients with pyometra these symptoms may, jointly or separately, supervene on the primary disease. At the same time, in the majority of pyometric dogs Borrensen (1984) diagnosed respiratory alkalosis, and metabolic acidosis was found only in a small proportion of such patients.

In multiparous animals (pigs) with uncomplicated farrowing the acid-base balance is characterized by great stability (Randall, 1982). The fact that out of the 6 dogs operated because of dystocia 3 had an expressed metabolic acidosis indicates that prolonged labour may lead to a shift of the acid-base balance towards metabolic acidosis. Further investigations are needed to determine the extent to which prolonged labour may influence the vitality of fetuses. The present investigations allow us to draw the conclusion that in dogs with pyometra, HGC or dystocia a shift of the acid-base balance towards metabolic acidosis should be reckoned with during operation.

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COMBINED SURGICAL AND RADIOTHYROIDECTOMY IN CHICKENS (RESEARCH NOTES)

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Thyroidectomized animals are widely used in order to better understand the physiological and pathological events governing thyroid economy.

In the chicken, a series of papers deal with thyroidectomy, both with surgical and radiothyroidectomy (Höhn, 1983). The question of whether total extirpation of thyroid activity can be achieved by these methods is raised from time to time, however. A paper recently published by Harvey et al. (1983), concerning the major problems of thyroidectomies, calls attention to the fact that, even with well-controlled surgical methods, disappearance of thyroid hormones from the blood plasma can hardly be attained. Several reasons might account for this phenomenon. The radiothyroidectomy, on the other hand, first suggested by Mellen and Wentworth (1962), had several side effects (Kapp and Pethes, 1971; Pethes and Fodor, 1975), thus interfering with some physiological processes.

In this paper a combination of surgical and radiothyroidectomy is described, which in many ways successfully unites the advantages of both techniques, eliminating their drawbacks.

Five groups of Hunniahybrid chickens were used. The animals were kept according to standard laboratory recommendations on a continuous lighting regime (24 h light) with food and water available ad libitum. Animals arrived at the laboratory when one day old. Four groups were radiothyroidectomized with increasing doses of carrier-free ^{131}I (NaI). The doses were as follows: 300, 600, 900, and 1200 μCi/100 g body mass, given intraperitoneally on day 2 of life, in 0.5 ml saline. On day 4, 7, 12, 17 and 21 of age the birds were killed and blood samples were taken in order to measure the concentration of thyroid hormones [thyroxine (T4) and triiodothyronine (T3) were measured according to Pethes et al., 1978*a*, *b*], and corticosterone (CRT). The latter was determined by competitive protein binding assay (Murphy, 1967).

One group was not treated with ^{131}I, but surgical thyroidectomy was performed in these birds according to a procedure described earlier (Rudas and Pethes, 1984). Ten birds formed one group throughout.

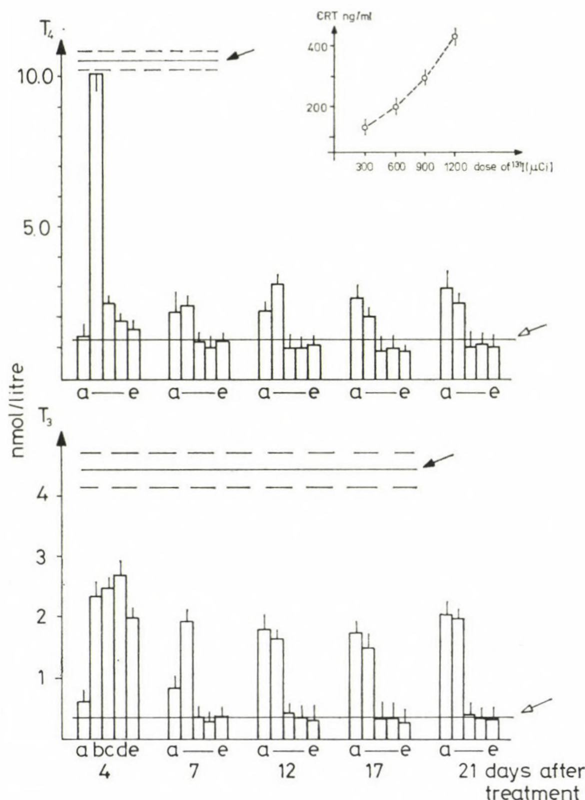

Fig. 1. **Serum concentration of T4 and T3 in surgically thyroidectomized (bars a) and radiothyroidectomized (bars b–e) chickens. Radiothyroidectomy was performed at several doses: b = 300, c = 600, d = 900 and e = 1200 μCi/100 g body mass ^{131}I (NaI) intraperitoneally. Full arrows indicate normal serum levels, open arrow stands for limit of detection. Upper right inset shows corticosterone (CRT) levels of chickens exposed to increasing amounts of ^{131}I**

In a separate experiment surgical thyroidectomy was performed on 10 animals, followed by 300 μCi ^{131}I (NaI)/g body mass intraperitoneally, with similar sampling as above.

Surgical thyroidectomy and thyroidectomy with 300 μCi ^{131}I alone did not depress thyroid hormone levels below the detection limit (Fig. 1). All the other groups, however, showed almost undetectable levels of thyroid hormones from 7 days after thyroidectomy. The inset in Fig. 1 demonstrates that the most stressing intervention was that of giving 1200 μCi ^{131}I intraperitoneally, namely the chickens in this group showed the highest plasma levels of corticosterone. Indeed, in this group losses were above 60 per cent within 3 weeks. The chickens in the groups receiving 600 and 900 μCi ^{131}I were of lower body mass than the surgically thyroidectomized ones, and developed severe lipid deposition in the liver, like that seen earlier (Kapp and Pethes, 1971) in ducks

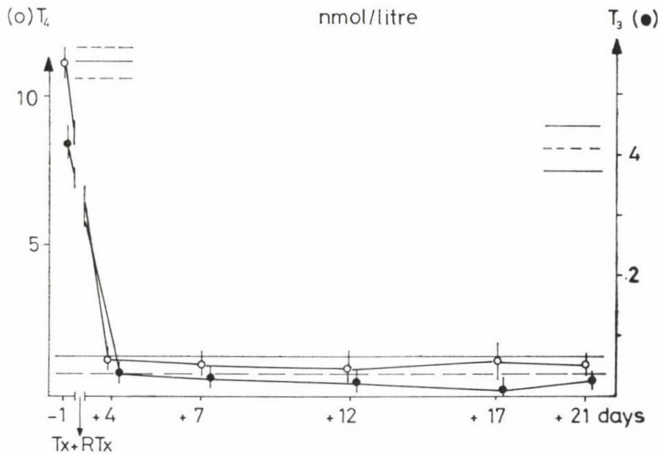

Fig. 2. The serum level of T4 (○) and T3(●) in chickens thyroidectomized with a combined method. Surgical thyroidectomy was followed immediately by 300 μCi ^{131}I per 100 g body mass. normal values for T4; normal values for T3; ——— detection limit for T4; - - - - - detection limit for T3

and geese (Pethes and Fodor, 1975). In the second experiment, which was done on the basis of the former, undetectable hormone levels could be attained (see Fig. 2), but no increase in serum CRT levels and no fatty liver was seen.

These experiments suggest that a combined surgical plus radiothyroidectomy (with low dose) are advantageous and will produce a good model for further investigation. With this method a series of experiments has been run in this laboratory. As a result of the latter, some ideas have been investigated concerning the protective mechanisms involved in adaptation to hypothyroidism in the chicken (Rudas and Pethes, 1985).

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DISTRIBUTION OF DEOXYRIBONUCLEIC ACID IN THE TESTES OF BUFFALOES, GOATS AND RAMS

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The distribution of deoxyribonucleic acid (DNA) was studied in the testes of buffaloes, goats, and rams. The distribution of DNA in spermatogenic cells was correlated to fourteen (I–XIV) specific stages of the seminiferous epithelial cycle (SEC). As compared to the haematoxylin-stained nucleus, the distinction of different cell types, especially of the different types of spermatogonia, was more clear in the DNA-stained nucleus. Based on distribution of DNA, three types of A spermatogonia (A_1, A_2, A_3), one type of intermediate spermatogonia (In) and two types of B spermatogonia (B_1, B_2) could be distinguished. The staining intensity and distribution of DNA in another category of spermatogonia, i.e. Ao spermatogonia, which remain constant throughout SEC were, more or less, similar to those of A-type spermatogonia. The distribution of DNA was also followed in different phases of meiosis. In spermatids, the condensation of DNA shows a step-specific phenomenon. DNA stainability also shows fluctuations during different phases of spermatogenesis. An interesting feature of the Sertoli cell nucleolus is the presence of one or more densely-stained DNA granules which decrease in size while increasing in number. In the tubular wall, only the external and internal cellular layers stain for DNA. In the Leydig cells, DNA stains intensely and contains fine granules. The results have been compared and contrasted to findings in other mammals.

Keywords. Deoxyribonucleic acid, distribution, testis, buffalo, goat, ram.

Daoust and Clermont (1955) localized the distribution of deoxyribonucleic acid (DNA) in the seminiferous tubules during the cycle of seminiferous epithelium in rats. As no other mammalian species has been investigated in this regard, the distribution of DNA in the rat seminiferous epithelium serves as a model. Since their study was completed, many authors, particularly Clermont (1967), Hilscher (1967), Hochereau (1967), Roosen-Runge (1969), Guraya and Bilaspuri (1976*a*) and Bilaspuri and Guraya (1980, 1984), have further divided different types of spermatogonia (A, In, B). Moreover, the details of cell types and their associations may vary with species; consequently, the results for rats may not be applicable to all mammalian species, especially in the farm animals, as (*i*) the distinction between different types of spermatogonia can easily be made in rodents in contrast to the farm animals (Clermont, 1972; Bilaspuri, 1978); (*ii*) the stages (1, 2, 3, 4, 8) of the 8-stage classification have been subdivided in the bull (Hochereau-de-Reviers, 1970), buffalo (Guraya and Bilaspuri, 1976*b*; Bilaspuri and Guraya, 1980), goat and ram (Bilaspuri, 1978), but such a subdivision is lacking in rodents (Bilaspuri and Guraya, 1984). Gledhill et al. (1966) demonstrated a dramatic difference

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in Feulgen staining associated with the differentiation of elongating and elongated spermatids into testicular spermatozoa. Keeping these points in view, the distribution of DNA in the testes and its behaviour during spermatogenesis have been studied in buffaloes, goats and rams. The last species is included as in our studies on this species the stages of the 8-stage classification were subdivided (Bilaspuri, 1978) and in contrast to 15 steps of acrosome formation (Clermont and Leblond, 1955), we could report 18 steps leading to the classification of seminiferous epithelial cycle into 14 stages which are not reported in any farm animal species (Bilaspuri, 1978).

Materials and methods

The testicular material from sexually mature buffaloes, goats and rams was collected as described earlier (Bilaspuri and Guraya, 1982). The tissue was put in 0.9% saline and cut into small pieces, which were immediately fixed in a sufficient volume of Zenker's formol and Carnoy's fixative (Humason, 1972). The tissue was processed as usual and was sectioned at 5 μm. For the distribution of DNA, the sections were stained with a modified Feulgen reaction and methyl green (Pearse, 1968).

Results

The distribution of DNA in various spermatogenic cells is diagrammatically shown in Plate 1 in relation to specific associations of cell types in stages I–XIV of the seminiferous epithelial cycle (SEC). The patterns of DNA staining in Sertoli cells, tubular wall and Leydig cells are given in Plate 2.

Spermatogonia. The slightly elongated nucleus of spermatogonia A shows very fine dust-like granules uniformly stained for DNA (Plate I). Sometimes, a few irregular granules may also be present. Comparatively, more intense reaction is observed along the nuclear envelope. Intense reaction is found around the nucleolus/nucleoli, which remain(s) unstained. In addition, some other areas in the nucleus are not stained for DNA. On the basis of distribution of DNA, three types of spermatogonia A (A_1, A_2, A_3) are recognized. The staining intensity of DNA towards the centre and along the nuclear envelope as well as the size of DNA-positive granules in these spermatogonia follow an increasing order from spermatogonia A_1 through A_2 to A_3.

Intermediate spermatogonia contain DNA-positive granules which further increase in size (Plate I). They are irregularly distributed throughout the nucleus and are attached to the nucleolus and nuclear envelope. The intensity of staining for DNA in these spermatogonia goes on increasing from stage 4a till they divide to form B_1 spermatogonia.

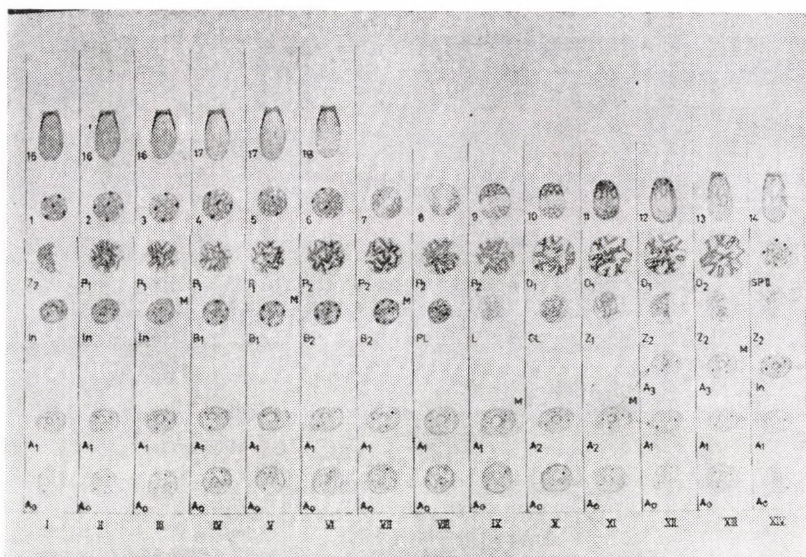

Plate 1. Diagrams showing distribution of DNA in spermatogenic cells during stages I–XIV of seminiferous epithelial cycle from buffalo testes. Zenker's formol fixation, Feulgen and methyl green stain. Similar results were obtained in goat and ram. Each column (I–XIV) indicates the distribution of DNA in spermatogenic cells in a given cellular association classified with PAS–haematoxylin technique. The Arabic letters show different steps of spermatogenesis. M denotes the stage in which spermatogonial mitosis takes place. For lettering see the list of abbreviations

The nuclei of B_1 spermatogonia, which tend to be spherical, have large DNA masses and granules attached to the nuclear envelope and the nucleolus (Plate I). The intensity of DNA staining and the size of granules further increase in B_2 spermatogonia as compared to the B_1 spermatogonia (Plate I).

The staining intensity and distribution of DNA in Ao spermatogonia, which remain constant throughout the SEC, are, more or less, similar to those of spermatogonia A (Plate I).

Spermatocytes. The nuclei of preleptotene spermatocytes stain more intensely than those of B_2 spermatogonia (Plate I). They have dark DNA granules which are relatively large and lie adjacent to the nuclear envelope and in the central nucleoplasm. In addition, fine DNA filaments are also present in the nucleus. In leptotene (stage IX), the spiral of DNA filaments is reduced to form thin, long and slender filaments (Plate I). In the "granular leptotene" (stage X), DNA filaments of leptotene begin to undergo contraction and spiralization, which result in the appearance of fine granules on them (Plate I). In zygotene (stages XI–I), DNA filaments, which are thick and made up of threads of coarse granules, occupy only a half of the nucleus to form a bouquet-like arrangement (Plate I). In early pachytene, the filaments are more or less similar to those of zygotene; however, they occupy a larger

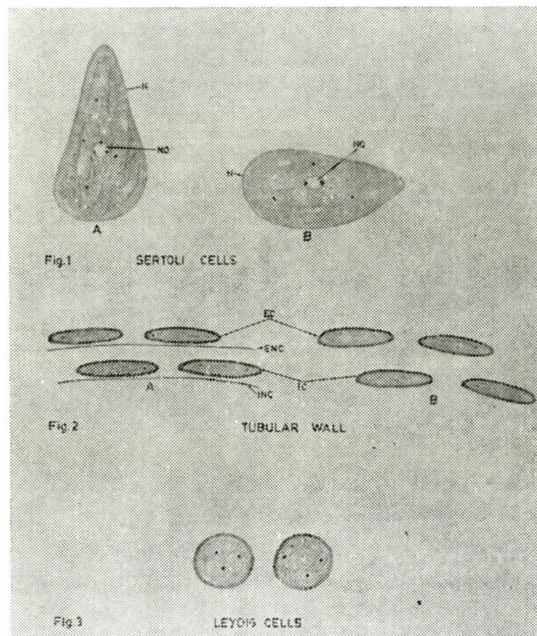

Plate 2. Diagrammatic sketches to show the pattern of DNA distribution in nuclei of different testicular components

Fig. 1. Two Sertoli cell nuclei showing their changing shape along the tubular wall in relation to the seminiferous epithelial cycle; the distribution of DNA remains unchanged. Note the presence of two or three DNA-positive dense granules in the unstained nucleolus

Fig. 2. A) Four layers of tubular wall (haematoxylin–eosin). B) In DNA staining only the external and internal cellular layers are seen; the non-cellular layers do not stain

Fig. 3. Leydig cell nuclei showing unstained eccentric nucleoli. The nuclei show different staining intensities on the periphery and in other regions

Abbreviations in plates. Ao: Ao spermatogonia; A_1, A_2, A_3: different types of A spermatogonia; B_1: B_1 spermatogonia; B_2: B_2 spermatogonia; D_1: early diplotene; D_2: late diplotene; EC: external cellular layer; ED: elongated spermatids; EL: elongating spermatids; ENC: external non-cellular layer; GL: granular leptotene; IC: internal cellular layer; IN: internal non-cellular layer; In: intermediate spermatogonia; L: leptotene; M: stage for mitosis; N_1 nucleolus; PL: preleptotene; P_1: early pachytene; P_2: late pachytene; 1–18: different steps of spermatogenesis

volume of the nucleus (Plate I). During the next phase of pachytene, the filaments are further thickening and shortening (Plate I). In the diplotene (stages X–XIII), the filaments disperse and tetrads are formed (Plate I). Due to the marked increase in the nuclear volume of the late pachytene and diplotene spermatocytes, there appear small spaces which do not stain for DNA (Plate I).

The secondary spermatocytes (stage XIV) have 5 or 6 large DNA granules of various sizes in addition to numerous fine granules, which are connected with one another by a fine filamentous network slightly staining for DNA (Plate I). Intense reaction is also seen along the nuclear envelope.

Spermatids. The distribution of DNA and the pattern of its condensation are similar to those of chromatin described for the development of spermatid nucleus (Bilaspuri and Guraya, 1985). However, the following differences are worthy of mention (Plate I). The filamentous network as well as fine and large granules present in spermatids (steps 1–9; Plate I) lightly stain for DNA as compared to the chromatin. The clear zone in steps 8–11, and vacuoles or lightly-staining cavities are well-marked in DNA staining as compared to the chromatin.

The staining intensity for DNA in round spermatids is lesser than that of premeiotic spermatogenic cells. This intensity first increases in elongating spermatids and then decreases in elongated spermatids and testicular spermatozoa.

Sertoli cells. Irrespective of the changes in shape and size of Sertoli cell nuclei throughout the SEC, the distribution of their DNA remains unaltered (Plate 2, Fig. 1). Among the diffused Feulgen-positive material, fine DNA granules are uniformly and lightly stained throughout the nucleus in addition to 2 or 3 small, spherical and intensely stained granules. However, irregular DNA-negative areas are also seen in the nucleus. The nucleus also contains DNA-positive filaments throughout, besides one or more nucleoli in the centre. Its periphery does not stain with either Feulgen or methyl green. In the nucleolus, there are one or more densely-stained DNA granules which decrease in size while increasing in number.

Tubular wall. Only the external and internal cellular layers of the tubular wall stain for DNA, the external and internal non-cellular layers remain unstained (Plate 2, Fig. 2). DNA along the nuclear envelope of fibroblasts stains intensely. The central region of the nucleus shows weak DNA staining; it may contain a few granules. Sometimes a few filaments are also seen.

Leydig cells. DNA staining is intensive along the nuclear envelope (Plate 2, Fig. 3). The central region of the nucleus stains weakly and contains fine granules. Two or 3 comparatively large granules are also seen. The filaments stain weakly. The region comparable to the eccentric nucleoli are unstained for DNA.

Discussion

In showing the dust-like granulations dispersed in the whole nucleus, an enhanced staining along the nuclear envelope and the presence of some regions in the nucleus free of staining for DNA, the present spermatogonia A resemble those described in the rat (Daoust and Clermont, 1955). However, a few irregular DNA-positive granules in the nucleoplasm and an intense reaction around the nucleolus/nucleoli were not described in the rat by Daoust and Clermont (1955). The large irregular masses along the nuclear envelope

reported by the same workers in the rat could not be demonstrated in the present studies. It is a further difference that no subdivision of spermatogonia A was made by Daoust and Clermont (1955) in the rat.

As compared to spermatogonia A, the intensity of DNA staining in In spermatogonia increases as also described by Daoust and Clermont (1955), who did not mention the variations of their DNA staining in different stages. The nuclei of B spermatogonia also show a comparatively intense staining but they do not give the cell a typical wheel-spoke appearance as described for the rat (Daoust and Clermont, 1955).

The small nuclei of preleptotene spermatocytes stain more intensely than those of the B_2 spermatogonia, but in the rat (Daoust and Clermont, 1955) they resemble the parent cells. The DNA filaments of the preleptotene spermatocytes have not been reported. The granular leptotene of this study was not reported by Daoust and Clermont (1955) in the rat. Instead of the bouquet-like arrangement of the DNA material in the zygotene, Daoust and Clermont (1955) reported coarse strands filling the whole nucleus. The small DNA-negative spaces which appear in the late pachytene and continue up to diakinesis are similar to the irregular Feulgen-negative interstices of the rat (Daoust and Clermont, 1955).

Instead of 4 or 5 large DNA granules, numerous such granules were reported in the rat, but the fine filamentous network lightly stained for DNA observed in the secondary spermatocytes was not reported in the comparable cells of the rat (Daoust and Clermont, 1955).

The increase in Feulgen stainability in steps 10–12 may be due to the reduction of nuclear volume by the change in the shape of spermatid nuclei, which becomes elongated (steps 10–12) compared to the spermatid nucleus (steps 1–9), but the masking of DNA by DNP has not yet started. The decrease in Feulgen stainability in steps 13–18 may be due to chromatin condensation, which starts at step 13 and may be attributed to arginine-rich basic proteins (Bilaspuri and Guraya, 1985). Similarly, a decrease in Feulgen reactivity and an increase in protein bound arginine, as shown by the Sakaguchi reaction, have also been reported in the bull (Gledhill, 1966; Gledhill et al., 1966). So the decreased Feulgen reaction may not be due to decrease in DNA content, as also suggested by Esnault and Nicolle (1976). Similarly, the reduction in the intensity of this staining for DNA in mammalian spermatozoa during epididymal passage (Bouters et al., 1967*a*, *b*) is not due to reduction in DNA quantity but appears to be the result of increased formation of disulphide bonds (Esnault, 1973).

The present observations on the Sertolian nuclear morphology and the variations in the regular nuclear shape during the SEC are similar to those reported by Daoust and Clermont (1955) and Fawcett (1975). In the distribution of the lightly-stained DNA granules dispersed throughout the nucleus

and the presence of some irregular Feulgen-negative areas, the Sertoli nucleus of the present species is similar to that of the rat (Daoust and Clermont, 1955), but the two to three small, intensely stained DNA granules and DNA-positive filaments seen in the Sertolian nucleus of the present species have not been reported by the above workers. On the whole, the nucleoplasm, which is remarkably homogeneous, may be similar to the electron-microscopically fine fibrogranular texture with a large proportion of euchromatin (Fawcett, 1975). The paucity of heterochromatin in the Sertoli cells is consistent with its being synthetically active and highly versatile in functions.

Feulgen-positive (one or more) densely stained DNA granules (heteropycnotic bodies) observed in the nucleolus of the present species may be comparable to the one or two intensely Feulgen-positive granules situated close to the nucleolus or lying in the nucleoplasm in rat (Daoust and Clermont, 1955). Similar heteropycnotic bodies were reported by Fawcett (1975). The physiological function of these bodies remains unknown. Unlike the reports of other workers (Nagano, 1966; Courot et al., 1970), no species-specific variations in the shape of nucleoli could be observed in the present species.

The cellular layers of the tubular wall, which stain for DNA in the buffalo, goat and ram, were not reported by Daoust and Clermont (1955). Similarly to the present studies, the intense staining on the periphery of fibroblasts was also reported for the rat (Daoust and Clermont, 1955).

The present findings have shown the architecture of different components of the testis as revealed by staining for DNA. The results obtained will form the basis for future studies on different aspects of spermatogenesis including the effects of different management conditions on the testes of these farm animals.

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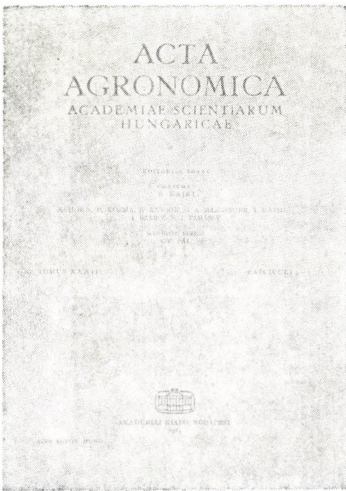

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COMPARATIVE STUDY OF *TREPONEMA* STRAINS ISOLATED FROM SWINE AND NUTRIA

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(Received September 15, 1985)

An infectious disease resembling swine dysentery (SD) was observed in a nutria stock. From the caecal mucosa of the succumbed nutrias large numbers of treponemes identical with *Treponema hyodysenteriae* in shape, size and motility were demonstrated microscopically. The *Treponema* strain was isolated from carcases of another nutria population. The strain of nutria origin was compared with *Treponema* strains derived from swine, by light- and electron-microscopic, biochemical and serological methods. Based upon its haemolysing ability, biochemical behaviour and swine-pathogenicity, the nutria-treponema resembled *T. innocens* rather than *T. hyodysenteriae*. However, after the third passage in pigs it proved to be pathogenic to swine.

Keywords. *Treponema*, nutria, swine, comparative study, swine dysentery, *T. innocens*, *T. hyodysenteriae*.

Earlier, *Treponema hyodysenteriae* was known to be a typically host-specific pathogen. As far as we know, there are no data indicating that, under natural conditions, it could produce disease in animal species other than swine.

In the dog, a disease resembling swine dysentery (SD) had been observed still earlier by Craige (1948), who attached importance to a spirochete in the aetiology of the observed condition; furthermore, he demonstrated similar organisms from diarrhoeic cats. It was established (Ulsen and Lambers, 1973; Adachi et al., 1979) that the spirochete causing "dog dysentery" (Craige, 1948) resembles *T. hyodysenteriae* not only morphologically but also in antigenic structure. However, after the dog-spirochete had been isolated (Turek and Meyer, 1977), it was found to be somewhat different from *T. hyodysenteriae* but practically identical with the slightly haemolysing *Treponema* strains isolated from swine; in one experiment this microorganism even colonized the large intestine of gnotobiotic pigs. Meier et al. (1982) isolated from dogs (in most cases from those with diarrhoea) treponemes of different properties, among them ones which were agglutinated by anti- *T. hyodysenteriae* serum. *T. hyodysenteriae* was isolated from the faeces of a dog which had ingested the blood-stained faeces of a dysenteric pig (Songer et al., 1978). In experiments of other researchers (Brandenburg et al., 1977) *T. hyodysenteriae* colonized the large intestine of gnotobiotic rats. Further studies revealed that *T. hyodysenteriae* was not only capable of colonizing guinea-pigs and mice, but it elicited in these species a non-haemorrhagic, mucous diarrhoea and appearance of gross and microscopic

lesions resembling those of SD (Joens et al., 1978; Joens and Glock, 1979). As compared to SD, there was an interesting difference, namely, in guinea-pigs and mice the pathological lesions developed primarily in the caecum. Based upon their further results, Joens et al. (1980) found the CF1 mouse strain suitable for differentiating between *T. hyodysenteriae* and *T. innocens*. When mice were experimentally infected with *T. hyodysenteriae* and accommodated in a way that their faeces fell on the pigs' feed, the pigs developed SD (Joens, 1980). Joens and Kinyon (1982) tested for the presence of *T. hyodysenteriae* wild mice and rats trapped in pig houses. *T. hyodysenteriae* strains were isolated from the caeca of 4 mice caught in 3 pig stocks affected with SD; of the strains, three proved to be pathogenic to swine. Two of the four mice exhibited gross lesions. Blaha (1983) isolated *Treponema* strains ("Borrelia" as called by him) from free-living wild rats, grey rats caught in cattle farms, and white laboratory rats; these strains were antigenically identical with *T. hyodysenteriae* and produced SD in SPF pigs. Blaha has concluded that mice are potential treponema-carriers, whereas rats can be regarded as a natural reservoir of the swine dysentery agent.

In Hungary, an SD-like disease was observed in a large-scale nutria stock (Sztojkov et al., 1982). Numerous microorganisms resembling *T. hyodysenteriae* were demonstrated, by direct immunofluorescence and staining, in the caeca of nutria carcases and in faecal samples collected from the diseased animals; their isolation, however, then failed. In the present paper the isolation of this microorganism and its comparison with *Treponema* strains of swine origin are reported.

Materials and methods

At necropsy of the two nutria carcases submitted to the Central Veterinary Institute for diagnostic purposes, the caecal mucosa showed superficial necrotic spots, pathological lesions identical with those reported previously, (Sztojkov et al., 1982). Treponema isolation was made on a selective medium containing spectinomycin (Songer et al., 1976). After isolation and purification the strains were maintained on an agar medium devoid of antibiotics. The electron-microscopic study of the *Treponema* strain isolated from nutria was performed after the 12th passage on agar, according to the method reported by Molnár (1979).

Biochemical studies

Treponema strains. Six strains, all our own isolates, were examined. Their main properties are shown in Table I.

In addition to the properties shown in Table I, we tested the strains for indole production and fructose, lactose, glucose, arabinose and maltose de-

Table I

Origin and main characteristics of *Treponema* strains used in the comparative studies

Designation of strain	Haemolysing ability	Swine-pathogenicity	Origin
S-2	strong	+	swine dysentery
S-10	strong	N*	swine dysentery
T-1	weak	—	swine; mass disease characterized by non-mucous, non-haemorrhagic diarrhoea
T-3	weak	N*	the same as for T-1
N-1	weak	—	nutria
N-2	weak	—	nutria

N*: not examined

composition. The *Treponema* strains were propagated in "Anaerobier—TVLS-Nährboden Basis" (Merck) broth containing 10% horse serum. Indole production was tested by adding Kovács's reagent to the cultures on days 2, 4, 6 and 8. Carbohydrate fermentation was studied in media in which the concentration of the given sugar was 1%; the test was considered positive if, after an incubation period of 5 days, the pH was at least by 0.5 lower than that of the control cultures. The cultures were kept at 37 °C in a mixture of 20% CO_2 + 80% H_2 gas, in the presence of palladium as catalyst.

Serological examinations

The agglutination and immunofluorescence (IF) tests were performed as described previously (Molnár, 1981; Molnár, 1983). Against strain S-2 we raised a hyperimmune serum in rabbits; this was used for preparing a conjugate to be used in the direct IF test, and was tested by agglutination against the antigens prepared from our six strains.

The hyperimmune serum produced against strain S-2 was used in the immunodiffusion test, too. The antigens were obtained as follows: the six strains were grown on the usual, tryptose-containing blood agar; 4-day-old cultures were washed with PBS and the treponemes were suspended in 0.5% sodium dodecyl sulphate solution; then the suspension was kept at 45 °C for 4 h (Adachi et al., 1979). Diffusion was made in 1% agarose.

Experimental infection

(1) From a nutria stock where no disease resembling SD had occurred and the pathogenic agent was not detectable in the faeces of animals, six growing (about 4 months old) nutrias were purchased. Two nutrias each were in-

fected with strain S-2 and N-1, respectively, whereas two served as control. The 4-day-old blood agar cultures of the respective strains were cut up into small pieces, mixed to small amounts of granulated feed, and this mixture was given to the animals at the morning feeding.

(2) The caeca of the nutrias from which the *Treponema* strains N-1 and N-2 had been isolated were cut up into small pieces and, together with the caecal contents, fed to two 8-week-old, treponema-free piglets, which were obtained by the method reported by Molnár (1981). A month later piglets were exsanguinated and their colons were fed to two further piglets. When one of the latter fell ill (on post-infection /PI/ day 21), its colon and the entire colon contents were mixed in the feed of three pigs. One of the pigs died on PI day 9; the two survivors were exsanguinated on PI day 30 and 45, respectively. Faecal examinations using the IF test were performed on every second day PI.

Results

When examined under phase-contrast microscope, and in stained preparations, the caecal mucosa scrapings of nutria carcases used for isolation contained moderate numbers of microorganisms resembling *T. hyodysenteriae* in shape and motility. In faecal smears fixed with acetone, using the conjugate prepated from the anti-*T. hyodysenteriae* (strain S-2) serum, moderate numbers of microorganisms exhibiting specific fluorescence and characteristic shape were seen (Fig. 1).

From the two nutria carcases successful treponema isolations were made on the selective medium. The first faint haemolytic zones appeared as late as on day 13 of incubation, whereas with strains isolated at the same time from pigs the characteristic haemolysis appeared as early as days 3–5. Upon further passage, the growth period gradually shortened and after the 6th–8th passage it was practically the same as that of *T. hyodysenteriae*.

By light and electron microscopy no difference whatsoever was found between *T. hyodysenteriae* and the nutria-treponema. In culture, also the latter had 2 to 4 large curvatures (Fig. 2) and its diameter was approximately 0.4 μm. There were 11 or 12 axial fibrils within a fibril bundle. Fibril bundles originated at both poles and overcrossed one another corresponding to the curvatures (Fig. 3) or ran side by side (Fig. 4). Fibril bundles crossed one another so many times as many curvatures the microorganism possessed (Fig. 2).

The results of the biochemical tests are given in Table II.

In the agglutination test performed with the anti S-2 rabbit serum the following titres were obtained with the different antigens: S-2: 2048; S-10: 1024; T-1: 1024; T-3: 512; N-1: 512; N-2: 512.

Each strain reacted positively with the 1 : 30 dilution of the S-2 conjugate in the direct IF test. The conjugate absorbed with strain T-1 or N-1 gave a

Table II

Biochemical properties of the *Treponema* strains

Designation of strain	Indole production	Fermentation of				
		Fructose	Lactose	Glucose	Arabinose	Maltose
S-2	±[1]	—	—	—	—	—
S-10	±	—	—	—	—	—
T-1	—	—	+[2]	+	—	—
T-3	—	+	+	+	—	+
N-1	—	—	+	+	—	—
N-2	—	—	+	+	—	—

[1] ±: very weak reaction (hardly observable even after 6–8 days)
[2] +: the pH of the culture is at least by 0.5 lower than that of the control tubes

positive reaction only with strain S-2 and even with this only in the undiluted state.

As shown by the immunodiffusion test, the swine and nutria strains possessed antigenic components in common; with the homologous S-2 antigen two, while with the other strains one intertwining precipitation lines were obtained.

In the first infection experiment, the nutrias remained healthy over a period of 8 weeks whichever strain was used. Attempts to demonstrate treponemes in their faeces failed.

In the second infection experiment, on PI days 4–6 treponemes appeared, although in very small numbers, in the faeces of piglets infected with the caecum of nutrias; nevertheless, the piglets remained healthy. The same held true for the second group. However, on PI day 21 one piglet of the third group developed diarrhoea, its faeces contained mucus and high numbers of treponemes. Since the piglet's condition did not change, two days later it was exsanguinated and its colon plus colon contents were fed to three piglets. On PI days 5, 7 and 8, respectively, the latter 3 piglets showed characteristic symptoms (mucous, blood-stained faeces) of SD; one of them died on PI day 9. In the colon of the succumbed pig and in that of pigs exsanguinated on PI days 30 and 45, respectively, there were pathological lesions characteristic of SD in varying severity, i.e. in the succumbed piglet, the mucous membrane of the colon was swollen, dark red and had a velvety touch; in the two exsanguinated piglets the mucosa of the colon showed scaly necroses in smaller or larger spots. The lesions contained masses of treponemes.

Discussion

Data of the literature have proved that *T. hyodysenteriae* is capable of colonizing the large intestine of species other than the pig. The question remains, however, whether under natural conditions it produces disease in these species.

Observations reported earlier (Sztojkov et al., 1982) and also our results indicate that the epizootic disease observed in nutrias, which is practically identical with SD as regards its clinical symptoms and pathological lesions, is caused by *T. hyodysenteriae*. No microorganisms resembling *T. hyodysenteriae* of could be demonstrated in healthy nutrias and in those affected with diarrhoea other nature; on the other hand, in the characteristic faecal samples taken from an affected stock and in carcases, such microorganisms were present in such large masses which rarely occur even in severe SD outbreaks. The chances of nutria populations contracting infection and the results of therapeutic attempts also support the involvement of *T. hyodysenteriae* in the aetiology of the disease (Sztojkov et al., 1982).

Our present results also speak in favour of the aetiological role of *T. hyodysenteriae*. We cannot explain the unusually long culture period needed for isolation; however, we observed even with *T. hyodysenteriae* strains, although very rarely, that at isolation the appearance of the first haemolytic zones took 8 to 10 days. Apart from this growth characteristic, by light- and electron-microscopical, biochemical and serological methods no difference was found to exist between *Treponema* strains of nutria and swine origin.

Kinyon and Harris (1979) assigned the strains non-pathogenic to swine into a new species and proposed for them the name *Treponema innocens*. With their strain P43/6/78 isolated from diarrhoeic pigs, Taylor et al. (1980) succeeded in producing the mild form of SD. They emphasized that this microorganism differed from *T. hyodysenteriae* in several respects and represented a new *Treponema* species. On the other hand, Blaha et al. (1984) are of the opinion that the "Borrelia" organisms colonizing the large intestine of pigs are pathogenic to the pig, but, depending on the degree of their adaptation to swine large intestine, they possess different virulence and always constitute a potential hazard, i.e. there exist no apathogenic "Borrelias".

The present studies do not allow us to take a definite stand on the proper place of strains isolated from the nutria, i.e. to determine what species they belong to. Namely, it seems to be contradictory that in its haemolysing ability, biochemical behaviour and swine-pathogenicity the nutria-treponema resembled rather *T. innocens* than *T. hyodysenteriae;* however, after the third passage in pigs it proved to be pathogenic to swine.

From results of the second series of experiments we can conclude that the *Treponema* strain isolated from the nutria is a *T. hyodysenteriae* variant slightly adapted to the nutria, which, however, recovered its swine-pathogenicity after a few pig passages. According to other observations of us, however, virulence cannot depend merely on the degree of adaptation, since *Treponema* strains with poor haemolysing ability are most frequently isolated precisely from stocks of long-standing infection (our own unpublished observations).

Further studies are needed to determine the relationship of the *Treponema* strains isolated from the nutria to swine *Treponema* strains of different virulence.

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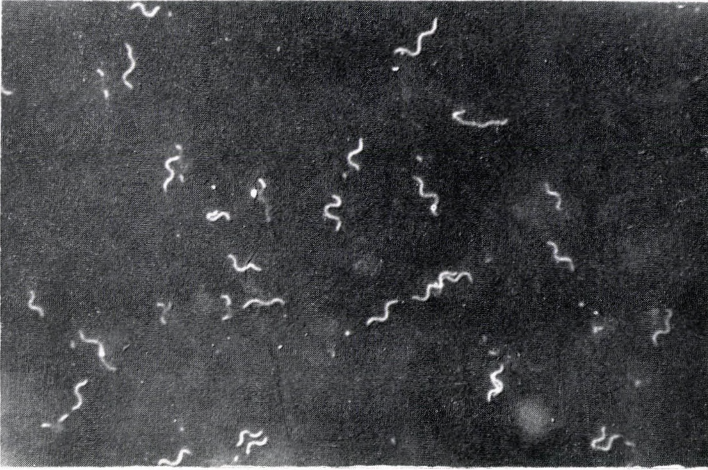

Fig. 1. Microorganisms of characteristic shape give specific fluorescence with the conjugate prepared from the hyperimmune serum raised against *T. hyodysenteriae*. Smear prepared from nutria faeces

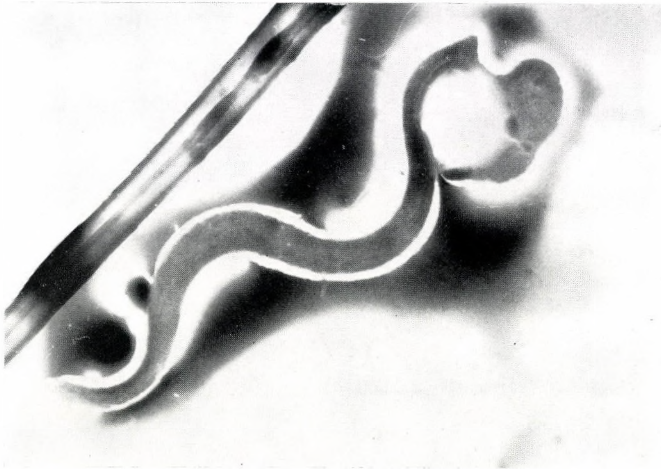

Fig. 2. Nutria-treponema possessing two great curvatures. EM, × 20,000

Fig. 3. Fibril bundles cross one another corresponding to the curvatures. EM, × 50,000

Fig. 4. Fibril bundles running side by side. EM, × 100,000

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EPIZOOTIOLOGICAL STUDIES ON PORCINE ATROPHIC RHINITIS X. STUDY OF THE HEAT-LABILE EXOTOXIN (HLT) OF *PASTEURELLA MULTOCIDA* IN MICE

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The effect exerted by the *Pasteurella multocida* HLT on the immune system and differential blood count of mice and, by double immunodiffusion, its immunological relationship with the *Bordetella bronchiseptica* HLT were studied.

The mouse lienotoxicity test was found to be suitable for determining the HLT-producing ability (toxigenicity) of *P. multocida* strains. Among mice intravenously infected with broth cultures of toxigenic *P. multocida* strains death occurred within a few hours due to the effect of the toxin. On the other hand, the spleen of the surviving mice remained unchanged over a period of 7 days. Contrarily, atoxic strains induce marked splenic hyperplasia and a manifold increase in the mass of the spleen, without giving rise to histopathological changes.

A positive correlation was demonstrated between HLT production and virulence of the strains.

The *P. multocida* HLT significantly altered the blood picture within 14 days, including a marked decrease of B lymphocyte counts. Contrarily, atoxigenic strains brought about a rise in B lymphocyte counts. The HLT of group-D *P. multocida* consists of 3 components, one of which is immunologically related to the homogeneous *B. bronchiseptica* HLT.

Keywords. Atrophic rhinitis, pig, *Pasteurella multocida*, heat-labile exotoxin (HLT), lienotoxicity test, *Bordetella bronchiseptica*.

Investigations into the aetiology and pathogenesis of porcine atrophic rhinitis (AR) have revealed that both the exotoxin-producing *Bordetella bronchiseptica* and *Pasteurella multocida* strains play a role in the induction of the disease (Éliás et al., 1985; Pedersen and Barford, 1981; Rutter and Rojas, 1982; Rutter, 1983). In eliciting the pathological process, the heat-labile exotoxin (HLT) produced by the strains is of decisive importance. Several researchers succeeded in eliciting in pigs' nose a regressive process characteristic of AR with HLT-containing sonicated bacterial suspensions of *B. bronchiseptica* or by applying purified *B. bronchiseptica* HLT on the nasal mucosa (Éliás et al., 1985; Hanada et al., 1979; Rutter and Mackenzie, 1984). Therefore, to judge the role played by the strains in the pathological process, we have to know their HLT-producing ability.

We know methods by which the degree of HLT production of *B. Bronchiseptica* strains (Éliás et al., 1983) and the HLT production of *P. multocida*

(de Jong et al., 1980; Rutter and Luther, 1984) can be determined. Further studies are needed, however, to get a closer insight into the properties of the HLT produced by *P. multocida* strains.

The objectives of our present studies were to determine (*i*) whether the mouse lienotoxicity test is suitable for assaying the degree of *P. multocida* HLT production; (*ii*) the effect exerted by *P. multocida* HLT on the lymphoid system and blood picture of mice; and (*iii*) the immunological properties of the HLTs of *B. bronchiseptica* and *P. multocida.*

Materials and methods

B. bronchiseptica and P. multocida strains

The two *B. bronchiseptica* (B-37, B-38), the four *P. multocida* strains belonging to Carter's serogroup A (219, 96, 1926, 6995) and the four *P. multocida* strains belonging to Carter's serogroup D (2248, 2296, 9949, 2599) were isolated from swabs taken from the nasal mucosa of 7 to 12 weeks old piglets exhibiting acute symptoms of atrophic rhinitis. The *B. bronchiseptica* strains were isolated on MacConkey agar, whereas the *P. multocida* strains on blood agar.

HLT studies

Determination of HLT production by the guinea-pig skin test. For the selection of *B. bronchiseptica* strains producing the HLT studied, 24-h cultures of numerous strains (viable cell count: 10^{12}) were subjected to sonication at 80 W, during cooling, for 4×0.5 min. The bacterial cultures were centrifuged at 14,000 *rpm* for 30 min, during cooling; 3 guinea-pigs each were inoculated intracutaneously with 0.1 ml of the clear supernate. The skin lesions were evaluated 48 h later (Éliás et al., 1982).

To determine the HLT production of *P. multocida* strains, 0.2 ml volumes of the strains' 24-h broth cultures were used, with which 3 guinea-pigs each were injected intracutaneously. The skin lesions were evaluated 48 h later (de Jong et al., 1980). With both the *B. bronchiseptica* and *P. multocida* strains, the evaluation of skin lesions took place according to the following key: $++$: the diameter of haemorrhagic inflammation or necrosis *(B. bronchiseptica)* exceeded 20 mm; $+$: the diameter of the inflammatory swelling was between 10 and 20 mm; $\pm$: the diameter of the inflammatory swelling was less than 10 mm; —: there was no pathological lesion at the site of injection.

Production of HLT fraction. B. bronchiseptica (B-37, B-38) HLT was produced by the method described above using the clear supernate obtained by sonication. *P. multocida* (strains D-2296, D-2248) HLT was prepared by centri-

fuging 48-h broth cultures at 14,000 *rpm* for 30 min. The bacterium-free filtrate of the supernate was concentrated tenfold in vacuo. The clear supernate *(B. bronchiseptica)* and filtrate *(P. multocida)* obtained by sonication were chromatographed on Sephacryl S-200 gel with saline (Éliás and Krüger, 1983; Van der Heijden et al., 1983). The HLT-content of the fractions was determined in the guinea-pig skin test.

Mouse experiments. **Inbred (CFLP) albino mice of 20 g body mass were used.**

In the first experiment five mice per group were injected into the tail vein, with 0.2 ml of the 10^{-1} to 10^{-4} dilutions of 24-h broth cultures (viable cell count: 10^9/ml) of the 8 *P. multocida* strains. Deaths were recorded over a period of 7 days; after the 7th day the relative spleen mass per 10 g body mass, of the surviving mice and those killed in ether narcosis, was monitored (Table I).

Table I

Demonstration of the heat-labile exotoxin (HLT) of *P. multocida* strains in the guinea-pig skin test and mouse lienotoxicity test

Strain	Sero-group	Guinea-pig skin test: Extent of necrosis after 48 h	Guinea-pig skin test: Number of guinea-pigs died within 12 h n/group = 3	Infection of 5 mice per group with the following dilutions of 24-h broth cultures: 10^{-1} D	10^{-1} S	10^{-2} D	10^{-2} S	10^{-3} D	10^{-3} S	10^{-4} D	10^{-4} S
219	A	++	2	4–5	—	5–5	—	1–4	51	0–2	57 (53–59)
96	A	+	2	5–5	—	3–5	—	0–2	64 (57–66)	0–0	65 (56–71)
1926	A	±	1	1–4	79	1–5	—	0–1	90 (78–96)	0–2	87 (82–92)
6995	A	—	0	0–5	—	0–3	144 -(136–152)	0–4	167	0–1	186 (151–197)
2248	D	++	2	5–5	—	3–4	56	2–4	63	0–1	81 (74–90)
2296	D	++	1	1–3	60 (54–66)	1–2	59 (51–63)	0–1	66 (55–70)	0–0	79 (71–93)
9949	D	+	1	1–2	62 (53–66)	0–1	58 (57–67)	0–1	84 (75–89)	0–0	97 (77–108)
2599	D	—	0	0–0	142 (117–165)	0–0	168 (114–196)	0–0	209 (150–245)	0–0	196 (161–249)
Control					54 (51–58)						

D: Deaths; the first figure indicates the number of mice died within 12 h, while the second that of mice died within 7 days

S: Relative spleen mass, mg/10 g body mass. In parentheses: the extreme values of spleen mass

In the second experiment, 10 mice per group were inoculated into the tail vein, with 0.2 ml of the 10^{-4} dilution of 24-h broth cultures (viable cell count: 10^9/ml) of 4 *P. multocida* strains (219, 2248, 2296, 2599).

Mice of one group received, also through the tail vein, 0.2 ml/day of the 15-fold diluted fraction of HLT produced from the filtrate of *P. multocida* strain D-2248 by chromatography. On PI day 14 heparinized blood samples were taken from the heart of 5 mice per group, of the surviving mice, and the leucocyte counts and, after Giemsa staining, the lymphocyte/leucocyte ratio were determined. Lymphocytes were separated on a Ficoll–Uromiro gradient (Böyum, 1968). After three washings in PBS, the cell suspension was incubated in RPMI 1640 medium (National Institute of Hygiene, Budapest) containing 10% calf serum, at 37 °C for 1 h to remove labile membrane immunoglobulins. After repeated washings, the B cells were demonstrated by direct immunofluorescence using FITC-labelled rabbit anti-mouse immunoglobulin (HUMAN Institute for Serobacteriological Production and Research, Budapest). The fluorescing cells were counted in a Leitz Orthoplan microscope (Table III).

Table II

Effect of the heat-labile exotoxin (HLT) of *Pasteurella multocida* on the mass of spleen, thymus and lymph node of mice, measured on the 14th day after intravenous infection in 5 mice per group*

Exotoxin production of strain (serogroup)		Spleen mean of relative mass (mg/10 g)	Thymus mean of relative mass (mg/10 g)	Mesenteric lymph node mean of relative mass (mg/10 g)
219 (A)	++	54 (52–59)	78 (72–90)	6
2248 (D)	++	69 (58–73)	87 (83–90)	7
2296 (D)	++	85 (74–92)	81 (75–87)	8
2599 (D)	—	214 (165–270)	142 (117–195)	17 (14–20)
HLT		51 (50–52)	80 (76–84)	7
Control		54 (51–58)	71 (64–78)	6

* In parentheses: extreme values

Table III

Effect of the heat-labile exotoxin (HLT) of *Pasteurella multocida* on the differential blood count of mice on the 14th day after intravenous infection*

Exotoxin production of strain (serogroup)		Leucocyte counts, mm³	Lymphocyte, %	Lymphocyte counts, mm³	B lymphocyte, %	B lymphocyte counts, mm³
219 (A)	++	300 (200–500)	65	195 (181–209)	14	27 (23–35)
2248 (D)	++	250 (194–325)	80	200 (180–226)	6	12 (8–16)
2296 (D)	++	200 (185–230)	61	122 (115–130)	12	14 (9–19)
2599 (D)	—	2800 (1750–3140)	80	2240 (1550–3010)	16	360 (245–510)
HLT		150 (142–160)	55	82 (72–96)	10	8 (6–11)
Control		1100 (970–1350)	57	625 (490–780)	11	71 (62–81)

*The values represent means obtained for 5 mice; in parentheses: extreme values

The spleen, thymus and a mesenteric lymph node of the mice were removed and, after their relative mass had been determined, fixed in 10% formalin. The histological sections were examined after staining with haemalaun and eosin (Éliás et al., 1983; Table II).

Antitoxin production and study of HLT relationships

The antitoxin was produced in rabbits. The HLTs of 2 *B. bronchiseptica* (B-37 and B-38) and 2 *P. multocida* (D-2296 and D-2248) strains were heated at 56 °C for 15 min. The rabbits were inoculated intravenously with 0.5 ml of the antigen at 3-day intervals over a period of 6 weeks. At the end of immunization, the antitoxin content of the serum was checked by agar gel precipitation using own HLT. The immunological relationships of HLTs produced by *B. bronchiseptica* and *P. multocida* strains were studied by double immunodiffusion performed in 1.5% Noble agar gels dissolved in veronal buffer of pH 8.6.

Results

Among the guinea-pigs and mice inoculated intradermally and intravenously, respectively, with toxigenic serogroup-A or serogroup-D *P. multocida* strains, some of the animals died within a few hours after injection. Deaths occurred considerably less frequently among the guinea-pigs and mice infected with less toxic or atoxic strains; this held true for both early deaths and the mortality occurring after several days. The spleen mass of mice infected with higher dilutions of the broth culture of toxic strains and surviving up to PI day 7 remained practically unchanged; however, that of mice infected with less toxic or atoxic strains underwent striking increase. The results are shown in Table I. Table II contains the results obtained by measuring, on PI day 14, the mass of organs of the immune system in mice intravenously infected with *P. multocida* strains and treated with HLT. Similar tendencies were revealed for other organs as for the spleen; namely, in mice treated with HLT or toxic strains the mass of the organs studied was almost identical with that of untreated mice, whereas in mice given the atoxic strain (2599) it reached striking sizes.

Changes of the differential blood count included a marked decrease in the number of leucocytes, first of all B lymphocytes, in mice treated with HLT or toxic strains, while in animals infected with atoxic strains there was a striking increase in leucocyte counts, as shown in Table III. Histopathologically no appreciable change was observed in the spleen of infected mice.

In the immunodiffusion test, with the *P. multocida* toxin (PT) the *P. multocida* antitoxin (PA) gave three precipitation arcs, whereas *B. bronchiseptica* antitoxin (BA) failed to give reaction.

Discussion

Earlier studies (Éliás et al., 1983) have revealed that the mouse lienotoxicity test is a suitable tool for demonstrating differences in the HLT-producing ability of *B. bronchiseptica* strains. Highly toxic strains severely damage the immune system; thus, the HLT of such strains results in regressive changes and a marked decrease in the mass of the immune organs, as well as a considerable reduction in the number of B lymphocytes within 7 days. Atoxic strains induce changes of an opposite trend, indicating that in this case the organism is superior to the toxin.

The results of our studies on *P. multocida* strains indicate that for determining HLT-producing ability the mouse lienotoxicity test can be used in another sense. In the case of toxic strains, in agreement with de Jong et al. (1980), numerous mice died shortly after treatment among those inoculated intravenously or intraperitoneally, due to the lethal effect exerted by the toxin. However, the spleen mass of mice infected with toxic strains but surviving up to PI day 7 remained almost unaltered. This fact suggests that the *P. multocida* exotoxin, consisting of several components, exerts an effect partly different from that of *B. bronchiseptica* exotoxin on the immune system of mice. The unchanged spleen mass is indicative of a strong toxigenicity of the strain, while the marked increase in spleen mass implies the lack of toxin production, i.e. the superiority of the organism over the toxin. The more toxic the *P. multocida* strain under study, the smaller the differences between the extreme values of spleen mass will be.

The susceptibility of guinea-pigs to the lethal effect of the toxin is reflected in the numerous early deaths observed among the animals infected intradermally. Other authors failed to observe this phenomenon, obviously because in the guinea-pig skin test they usually injected the sterile filtrate of smaller HLT content instead of the bacterial culture. The skin reaction of smaller extent observed by them (de Jong et al., 1980) is consistent with this.

The resemblances existing between the biological properties of the *P. multocida* HLT and *B. bronchiseptica* HLT are reflected in the fact that both resulted in a marked decrease in the B lymphocyte counts of mice.

Between the HLT production and virulence of *P. multocida* strains there is a positive correlation similar to that reported for *B. bronchiseptica* strains: the more toxic the strain was the more deaths occurred among the inoculated mice. This fact shows that the HLT is a very important virulence factor of *P. multocida* strains as well (Éliás et al., 1983).

The *P. multocida* HLT was produced from the bacterium-free filtrate of broth cultures by chromatography. Toxin fractions gave distinct peaks in the chromatogram, suggesting that they did not contain other antigens of cell origin. In agreement with the results of Van der Heijden et al. (1983), we estab-

lished that the HLT of serogroup-D *P. multocida* consisted of 3 components, none of which was related to the *B. bronchiseptica* HLT which had been shown to be homogeneous in our earlier studies (Éliás and Krüger, 1983).

Although the present studies comprised two *B. bronchiseptica* HLTs and two serogroup-D *P. multocida* HLTs, the results of immunological investigations can be generalized, since in earlier studies (Éliás and Krüger, 1983) we elucidated the identity of the HLT of *B. bronchiseptica* strains and, in agreement with us, Van der Heijden et al. (1983) established that the HLT of *P. multocida* strains consisted of several components.

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A NEW SYNTHETIC, SELECTIVE, LIQUID MEDIUM FOR DETERMINATION OF COLIFORM BACTERIA

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A selective liquid medium, composed of synthetic constituents, was developed for the demonstration and determination of coliform bacteria. The medium, containing only the minimum nutrients for coliforms, does not support the growth of other microorganisms.

The synthetic medium acting on a new principle is of the same sensitivity and reproducibility as the brilliant green–lactose–bile (BLB) broth, the medium generally accepted in international food-inspection practice, and has several advantages over the old one. Furthermore, the usability of the new synthetic medium does not depend on the accompanying flora of the material to be tested; e.g. meat, milk, dairy products or even water can equally be tested. The same does not hold true for all of the previously used media.

The new medium differs in composition from that specified by the international regulations concerning the assay of coliform bacteria in so far as it does not contain bile or bile salts. It can easily be prepared in powder form and can be stored over a long period. It is easy to prepare: it does not require filtration or pH adjustment, only the components need to be suspended. Therefore, the new medium would be particularly suitable for use in a uniform, centralized medium supply system.

Keywords. Coliform, bacterium, selective, differential medium, synthetic, inhibitory substance.

In food microbiology the qualitative and quantitative determination of the so-called indicator flora is a commonly used procedure which allows us to draw, rather rapidly and easily, conclusions concerning possible contamination of a product in general, and a contamination by pathogenic agents in particular. Of the indicator microbes, coliform organisms are used most frequently. Their presence is indicative of external contamination. The assay of coliform bacteria in foods, feeds and water is included as a testing method in food microbiology.

The determination and enumeration of coliform bacteria are governed by Hungarian and international standards and regulations (Hungarian Standard 3640/17-79; Hungarian Standard 3743/1-78; International Standard ISO/DIS 4831.)

Numerous selective and differential media are being used for the determination of coliforms. Their common basic principle is that to the broth containing protein hydrolysate(s) (e.g. peptone) inhibitory substances are added which prevent the growth of gram-positive bacteria. As inhibitory substance, usually

crystal violet or brilliant green is used. To inhibit the growth of aerobic sporogenic microorganisms, the media contain ox-bile, too. After an incubation period of 48 h, coliforms induce gas formation in the medium.

The evaluation of media on the basis of gas production may be hampered and disturbed by the presence of proteolytic bacteria in the sample to be tested, since these produce gas not from lactose but from peptones. Therefore, in the case of a moderate gas formation, the standards relating to the question ordain a subculture on a solid selective medium to show whether the cultured microbe produces acid or not. This, of course, delays the diagnosis with further 24 h; however, its omission may lead to false results.

Despite the application of inhibitory substances, several gram-negative, non-coliform microbes are able to grow in/on selective media. Some of these bacteria might inhibit the growth of coliforms solely by their presence. Finally, it should be mentioned that the inhibitory substances are not completely indifferent to the very bacteria that are to be demonstrated. According to data of the literature and our own experience, ox-bile used as an inhibitory substance may inhibit the growth of coliforms.

The efficiency of liquid and solid media used in these studies has already been compared by us in Hungary (Szita et al., 1979; Szita et al., 1981). It was found that although the hitherto applied media may inhibit the growth of the bacteria to be demonstrated, the concentration of inhibitory substances cannot be reduced considerably without counteracting selectivity. Therefore, the efficiency of media containing inhibitory substances cannot be improved.

Materials and methods

Since the disadvantages of the selective and differential media used so far (brilliant green–lactose–bile (BLB) broth, Kessler–Swenarton's broth and MacConkey broth) cannot be eliminated, we have evolved a medium functioning on a new principle. In essence: it contains only those inorganic salts (nitrogen source), carbon source and trace elements which are just sufficient for the growth of coliform bacteria but do not support the growth of other microbes. In this way we can achieve a very good selectivity in an indirect manner, without using an inhibitory substance. The medium was named, and is hereinafter referred to as, X broth.*

In the present work we performed a comparative study of the new medium. The materials used, the medium used for comparison (BLB broth) and the test method were consistent with those stipulated for in the Hungarian Standard 3743/1-78.

* The new synthetic medium is currently under patenting in Hungary.

To check its efficiency, the X broth was compared with the BLB broth specified in both Hungarian (Hungarian Standard 3743/1-78) and international (International Standard ISO/DIS 4831) standards. In the first step, both media to be tested were inoculated with the coliform *Escherichia coli*, *Klebsiella pneumoniae* and *Enterobacter aerogenes* strains, with the non-coliform but gram-negative *Proteus mirabilis*, *Salmonella enteritidis* and *Pseudomonas aeruginosa* strains and, of the gram-positive bacteria, with *Bacillus cereus*, *Staphylococcus aureus* and *Micrococcus* strains.

We compared the sensitivity of the two media for coliforms, using the "most probable numbers (MPN)" method described by Hoskins (1934). From dilutions of the test samples 3 parallel inoculations each were made into both media. To identify and remove possible methodological errors, 2×3 parallel inoculations were made from both media. In the first step the pure cultures of *K. pneumoniae* and *E. coli* were used as a model. The use of model experiments rendered possible to exclude the complicated interactions (synergism, antagonism) occurring in mixed microflora. Subsequently, we determined the coliform count of camembert cheeses of highly mixed microflora, using our media. The results were evaluated by mathematical and statistical methods (Weber, 1971).

Results

The *E. coli*, *K. pneumoniae* and *E. aerogenes* strains grew well and produced gas in both media. However, the other gram-negative bacteria, although grew well in the BLB broth, failed to produce gas. In the X broth non-coliform microbes showed no growth whatsoever even if the cultivation period was prolonged over several weeks. The gram-positive bacteria tested caused turbidity, without producing gas, in the BLB broth and did not grow at all in the X broth.

The biometrical evaluation of results obtained by the MPN method indicates that the BLB broth and X broth are identical in sensitivity, and the methodological error of the determinations is statistically identical, too. Here we do not enter into the details of the statistical calculations.

The results of model experiments conducted with pure cultures are shown in Fig. 1, whereas those performed with the camembert cheeses Tihany and Bakony in Fig. 2. Both figures indicate that there was no significant difference between the coliform counts obtained with the two media neither in the case of pure cultures nor in that of the camembert cheeses.

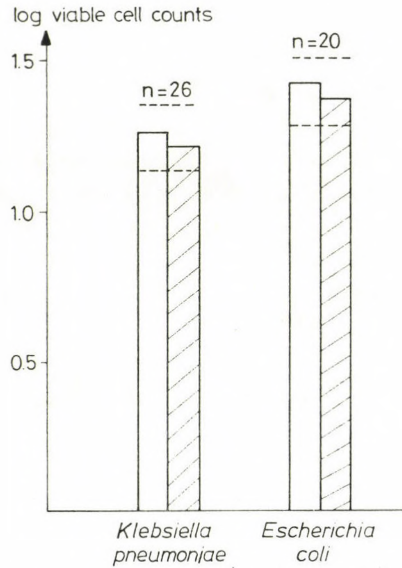

Fig. 1. **Comparison of the brilliant green–lactose–bile (BLB) broth and the X medium in model experiments. White columns: BLB broth; striated columns: X broth**

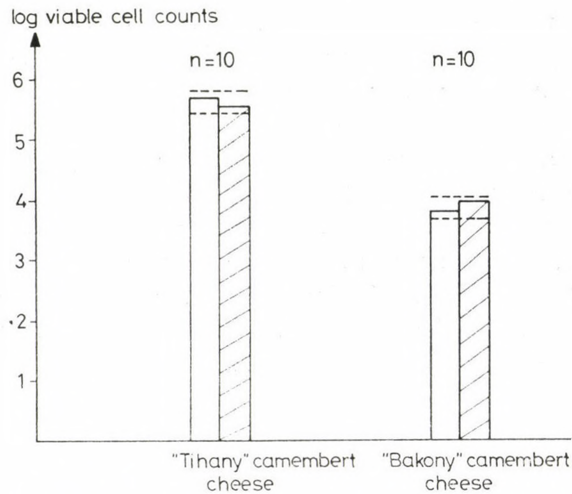

Fig. 2. **Comparison of the brilliant green–lactose–bile (BLB) broth and the X medium in the examination of camembert cheeses. White columns: BLB broth; striated columns: X broth**

Discussion

The favourable experiences gained with the new medium can be summarized as follows.

(1) It does not require the use of inhibitory substances; thus, there is no incomplete inhibition and the growth of the cultured microbe is not inhibited either.

(2) In the X broth bacteria other then coliforms do not develop— thus, the medium is highly specific and no antagonism has to be reckoned with.

(3) Owing to the clearness and transparency of the medium, its evaluation is easy and rapid.

(4) No further subcultures are needed for an accurate diagnosis; thus, the test is less time-consuming.

(5) The medium is easy to prepare: only the necessary salts have to be suspended; there is no need even for filtration.

(6) Less intensive heat-treatment is needed for the sterilization of the X broth, since the inorganic salts are less abundant in germs than the peptones, and are devoid of proteins which could exert a protective effect on bacteria.

(7) The medium is easy to produce in powder from.

(8) Its production cost is a small portion of that of conventional media, since expensive ingredients like meat paste, peptone and bile are not required.

(9) Its composition is standard and easily reproducible anywhere and at any time.

(10) It can be stored over very long periods since the decomposition of proteins and a possible concentration of the inhibitory substance need not be reckoned with (the X broth worked excellently even after stored at room temperature over a period of 6 months).

It can be concluded that this new selective broth would be suitable for the purposes of a uniform, centralized medium supply service.

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APPLICABILITY OF COAGGLUTINATION TEST FOR DETECTING *AEROMONAS SALMONICIDA* ANTIGENS FROM EXTRACTS OF BACTERIAL CULTURES AND EXPERIMENTALLY INFECTED TISSUES OF THE COMMON CARP *(CYPRINUS CARPIO* L.*)*

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Attempts were made to adapt the coagglutination test for identification of an "atypical" *Aeromonas salmonicida* variety, the bacterium causing carp erythrodermatitis (CE) in Europe. The test is based on detection of the pathogen's antigen.

Rabbit antisera raised with two "atypical" and a typical strain of *A. salmonicida* were adsorbed to formalin-fixed *Staphylococcus aureus* bacteria producing protein A and bacterium suspensions thus obtained were used as serological reagents in coagglutination.

Extracts of tissues of carp infected artificially and heat-treated broth cultures of fish-pathogenic strains of the genera *Aeromonas, Pseudomonas, Vibrio, Edwardsiella* and *Streptococcus* were used as antigens.

The coagglutination test proved to be a sensitive tool for detecting antigens of *A. salmonicida* in infected tissues.

Cross reactions did not occur except with weak late reactions with some of the control tissue extracts and the antigen of *A. hydrophila* ssp. *anaerogenes.*

The test is considered to fit the requirements of diagnostic work better than bacterium isolation or immunofluorescence.

Keywords. Coagglutination test, *Aeromonas salmonicida,* antigen, erythrodermatitis, common carp (*Cyprinus carpio* L.).

Carp erythrodermatitis (CE) is a disease characterized by ulcers on the body surface of several cyprinid species. It may be fatal up to around 25% in pond culture, and still more fish of affected stocks cannot be marketed for noticeable signs of ongoing or past disease. The causative agent is a variety of *Aeromonas salmonicida* (Bootsma et al., 1977), a bacterium poorly categorized taxonomically. It is very hard or even impossible to clinically differentiate CE from ulcerative diseases originating from other bacteria (Schulz and Bulling, 1981; Heuschmann-Brunner, 1978).

Laboratory diagnosis has been based on culturing the causative agent from the ulcers; for its identification its most important morphological and biochemical characteristics were taken into account, though direct agglutination of heat-treated bacterial cells is also possible. Culturing of the bacterium,

however, is not easy because accompanying members of the bacterial flora can quickly overgrow *A. salmonicida*. Furthermore, culturing of the CE agent is time-consuming, it lasts four or five days.

A faster and efficient diagnosis is facilitated by detecting the causative agent or its antigens in the fish tissues by immunological methods. A direct immunofluorescence method, evolved by Wiedemann (1981), is very sensitive but requires great expertness and an appropriate equipment.

The coagglutination test, another sensitive test method, on the other hand, does not demand well-trained personnel and expensive tools. The test is based on the specific reaction between antibodies bound to the surface of stabilized *Staphylococcus aureus* and homologous antigens present in the fluid examined. The resulting coagglutination can be observed by the naked eye, or by low magnification under a dissection microscope. The antibodies are bound to the protein A linked covalently to the cell wall of *S. aureus* (Forsgren and Sjoquist, 1967). The test was used first by Kronvall (1971) for bacterium typing; it was introduced in the diagnosis of fish diseases by Newman et al. (1981). In this work with salmonids, the coagglutination test was used successfully to specifically detect *A. salmonicida* antigens, in accordance with the results of Kimura and Yoshimizu (1983).

Little is known of the antigenic characteristics of the variety of *A. salmonicida* causing CE. Bootsma and Blommaert (1978) as well as Wiedemann (1979) made a comparison of some of the antigens of their isolates; the antigens proved to be of identical nature. Sövényi et al. (1984) claimed that ten "atypical" *A. salmonicida* strains isolated in different parts of Hungary had identical heat-stable antigens detectable by immunoelectrophoresis. Cross reaction can occur between *A. salmonicida* and some strains of the *A. hydrophila* group (McCarthy and Roberts, 1980).

The aim of the present work was to examine the sensitivity and selectivity of the coagglutination test from the point of view of the diagnosis of CE.

Materials and methods

Preparation of bacterium suspension and its adsorption were made by the method described by Newman et al. (1981), with minor modifications.

Staphylococcus suspension. S. aureus ATCC 12958, a good protein-A-producer, was cultured in brain heart infusion broth (Difco). Its 48-hour culture was centrifuged and killed with 0.3% neutralized formalin. Bacterial bodies were washed five times in PBS (pH 7.4) and stored in PBS with 0.02% sodium azide until used.

Rabbit anti-A. salmonicida sera. Forty-eight-h cultures were freshly made for each injection from the "atypical" strains H2 and S20, strains isolated from

diseased carp as well as from the typical *A. salmonicida* ATCC 17147. A suspension containing 40 mg live bacterium/ml PBS was made of the culture. Each suspension was injected into two New Zealand white rabbits intradermally in the area above the blade four times, at two-week intervals. Ten days after the last injection, 20 ml of blood was withdrawn from each rabbit. The antisera thus obtained were titrated by passive haemagglutination (Sövényi et al., 1984). The titres were $\geq$ 1 : 4096. The homologous sera were pooled and preserved in the presence of 0.02% sodium azide.

Absorption of antibodies to staphylococci. A 0.2-ml centrifuged *Staphylococcus* pellet was resuspended in ten volumes of serum and incubated at room temperature for one day with slow stirring. The bacterial bodies were washed five times in PBS and were finally resuspended to form a 1% suspension. The reagents were stored in the presence of thymol crystals at 4 °C.

Infection of carp and sample preparation. The "atypical" strain S20 was grown on blood agar. Its 48-h growth was collected and suspended in sterile 0.65% saline. Turbidity was adjusted to 0.1 spectrophotometrically at 525 nm. From the bacterial suspension, 25 μl was injected subepidermally into the dorsolateral area of each of 32 mirror carp averaging 27.2 g body mass. The fish were kept in 18 °C water. The local reaction was judged on the seventh day after inoculation. The diagnosis was "ulcer" when there was an epithelial destruction connected with the inflammatory process. Local inflammation marked by circumvented red zone was denoted "inflammatory". In the absence of red spot the reaction was regarded as "abortive". For control, 20 carp of identical origin were injected in the same way with saline. The area surrounding the site of injection, 2 cm in diameter, was excised. The excised tissue, including muscle, was homogenized in 5 ml 2/3$\times$PBS and centrifuged for 10 min at 1000 *g*. The supernatant was tested for *A. salmonicida* antigens.

Other bacteria. Antigens were made from broth cultures of the following bacterium strains. "Atypical" strains isolated from CE: H2, OAI13, S19, S20, V75/175, V75/176, CEO1; typical strains of *A. salmonicida:* ATCC 17147, IP 6450; *A. salmonicida* ssp. *masoucida* NCMB 2020 (masou salmon, Kimura, 1969); *Aeromonas hydrophila* ssp. *hydrophila* 831, 832, 848 (from skin ulcers of carp); *A hydrophila* ssp. *anaerogenes* H-34; *A. punctata* ssp. *punctata* 602, 623 (from the liver of carp); *Pseudomonas* sp. 966; *Streptococcus* sp., D serotype NH-1 (from gill necrosis of carp, secondary infection; Farkas and Oláh, 1981); *Str. faecalis* ssp. *zymogenes* 1973 (rainbow trout); *Edwardsiella tarda* EF-1 (eel); *Vibrio* sp. (European catfish fry; Farkas and Malik, in press); *Vibrio anguillarum* NCMB 6 (eel.) Cultures of these bacteria were processed for the coagglutination test according to Kimura and Yoshimizu (1984).

Coagglutination. One drop of the fluid to be tested was mixed with the same volume of coagglutination reagent on a slide. The reaction was checked after 30, 60 and 120 min of incubation in a moist chamber at room temperature. The

coagglutination reaction visible by the naked eye was considered positive; that visible under the dissection microscope intermediate, and no coagglutination was registered as negative.

Results

The results of artificial infection and the coagglutination tests in connection with it are summarized in Table I. All fish having ulcer gave positive coagglutination with all of the anti-H2, anti-S20 or anti-ATCC 17147 sensitized *Staphylococcus* suspensions.

The samples from carp of "inflammatory" and "abortive" reaction had a tendency to cause coagglutination progressing with the time. However, development of coagglutination was the fastest right after mixing the reagents.

Table I

Coagglutination test with tissue extracts from artificially infected carp and those from the negative control

Local reaction, coagglutination	Incubation time		
	30 min	60 min	120 min
"Ulcerative"			
positive	15	15	15
"Inflammatory"			
negative	2	1	—
intermediate	2	2	2
positive	4	5	6
"Abortive"			
negative	4	4	3
intermediate	2	1	2
positive	3	4	4
Negative control			
negative	20	17	17
intermediate	—	3	1
positive	—	—	2

Control tissue samples did not coagglutinate sensitized staphylococci during the first 30 min. Later, three samples turned intermediate and two shifted to the positive coagglutination category.

Table II demonstrates the coagglutination test of bacterial culture extracts. All strains belonging to *A. salmonicida* species gave positive coagglutina-

tion with all the three sensitized *Staphylococcus* suspensions. The antigen extract of *A. hydrophila* ssp. *anaerogenes* induced late coagglutination.

The clear culture supernatant of typical *A. salmonicida* strains was positive in the coagglutination test on the second or third day after inoculation. CE agents took usually longer time to release antigenic substance enough for positive coagglutination.

Table II

Coagglutination test with culture extracts from some of the important disease agents of fish, read after 30-min incubation

Bacterium strain examined	Sensitized suspension		
	a-H2	a-S20	a-ATCC 17147
A. salmonicida, "atypical" strains			
H2	+	+	+
OAI13	+	+	+
S19	+	+	+
S20	+	+	+
V75/175	+	+	+
V75/176	+	+	+
CEO1	+	+	+
A. salmonicida ssp. *salmonicida*			
IP 6540	+	+	+
ATCC 17147	+	+	+
A. salmonicida ssp. *masoucida*	+	+	+
A. hydrophila ssp. *hydrophila*			
831	—	—	—
832	—	—	—
848	—	—	—
A. hydrophila ssp. *anaerogenes*	—(+)*	—(+)	—(+—)
A. punctata ssp. *punctata*			
602	—	—	—
623	—	—	—
Pseudomonas sp.			
966	—	—	—
Streptococcus sp.			
NH-1	—	—	—
Str. faecalis ssp. *zymogenes*	—	—	—
Edwardsiella tarda			
EF-1	—	—	—
Vibrio sp.			
Ae110	—	—	—
Vibrio anguillarum			
NCMB 6	—	—	—

* In parentheses, coagglutination after 120 min.

Discussion

One of the aims of our study was to examine the sensitivity of the coagglutination test for the CE agent. We kept in mind the possible use of the test in routine diagnosis since it has proved useful in field examinations for the diagnosis of fish furunculosis, an analogous disease of salmonids (Kimura and Yoshimizu, 1983). Antigen detection from artificially-infected tissues shows that the amount of antigen produced in all the carp having ulcer sufficed for a fast and expressed coagglutination. In these experimental conditions, an incubation for one week was enough for the development of ulcers not exceeding 5 to 6 mm in diameter and being of little depth (not reaching below the corium). Such cases are not subject to examination in diagnostic laboratories in the practice, where diseased fish showing deep and late, subacute or chronic ulcers are examined usually. The latter are supposed to be more abundant in the antigens in question. The sufficiency of the sensitivity of the coagglutination test for the CE agent is underlined by the fact that several "inflammatory" and "abortive" tissue samples were positive.

In some of the positive or late coagglutination reactions of tissue samples, tissue antigens may have also had a role, as indicated by the results with control samples. Newman et al. (1981), using an *A. salmonicida*-sensitized *Staphylococcus* suspension, noted a similar phenomenon with salmonids.

Selectivity of the test was checked with bacterium strains of identical or different species of which some are, others may potentially be, fish pathogens in Hungary.

It has been known for a long time that the heat-stable antigens of the typical strains of *A. salmonicida* are identical in the most isolates, only partial differences were detected (Karlsson, 1964; Popoff, 1969; McCarthy and Rawle, 1975; Hahnel et al., 1983). This may be explained by the homogeneous and simple biochemical composition of the O antigen (Shaw et al., 1983). "Atypical" strains isolated outside Europe and *A. salmonicida* ssp. *masoucida* show no essential serological difference from typical strains (Kimura, 1969; McCarthy, 1975; Paterson et al., 1980). On the basis of recent data, the same is true for the CE agent (Bootsma and Blommaert, 1978; Wiedemann, 1979). The results of the coagglutination test in this study are in accordance with the above data, since cross reaction between non-homologous antigens of *A. salmonicida* species and anti-*A. salmonicida* antibodies proves the presence of a major common antigen. Therefore, the coagglutination test in this layout is not suitable for differential diagnosis of CE agent vs. other *A. salmonicida* varieties. For this purpose, bacterium isolation remains the safe solution.

Cross reaction between the antigens of "atypical" strains of *A. salmonicida* and *A. hydrophila* did not appear by the 30th min of incubation; later, positive reaction developed with one of the strains used. This corresponds to the results

of a comparative serological study on typical strains and some *A. hydrophila* strains. With the majority of the strains, cross reaction does not occur (McCarthy and Rawle, 1975), or weak cross reaction can be observed between certain strains (McCarthy and Roberts, 1980). An *A. hydrophila*, an *A. punctata*, and an *A. liquefaciens* strains was included in Kimura's (1983) work on the coagglutination test. He used *S. aureus* suspension sensitized with anti-(typical) *A. salmonicida* and saw no coagglutination after 30 and 60 min.

However, the possible cross reaction originating from *Aeromonas* species other than *A. salmonicida* is not considered to be important in the application of the coagglutination test for CE, because coagglutination inside the *A. salmonicida* species always completed in 30 min, mostly in less than 15 min; the reactions were readily noticeable. Naturally, in case of ambiguity, the result of a coagglutination can be checked by isolation of the pathogen. The same holds for cross reactions with tissue antigens.

The heat-stable antigens of typical *A. salmonicida* strains did not cross-react with antigens of *Pseudomonas fluorescens* and *Vibrio anguillarum* in the passive haemagglutination test (McCarthy and Rawle, 1975). Negative reaction was demonstrated with the antigen originating from *Vibrio anguillarum* I, II biotype in coagglutination tests performed by Newman et al. (1981) and Kimura (1983). The present results are in full accordance with that of the authors cited in this paragraph. Furthermore, adsorbed antibodies produced with the CE agent did not couple with any of the antigens outside the *Aeromonas* genus (Table II). These facts strongly support the assumption that, practically, there is no need to reckon with cross-reaction with bacterial antigens originating from an agent presently occurring in Hungary or one expectable in the future.

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SKELETAL DEVELOPMENT IN RUMINANTS: FURTHER DATA ON SEXUAL DIMORPHISM IN ELK *(ALCES ALCES)*

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To the memory of Dr. Gy. Fábián

The analysis of relative growth tendencies in the interpretation of hormonal changes has most widely been applied to domestic animals. In the case of cattle and sheep such studies help better understanding of meat production. The overlap between the effects of breed, age and sex, however, usually tends to blur the picture.

In this study, a special set of data was used to illustrate the sexual dimorphism of skeletal growth in relation to the rest of the body. Metacarpal measurements as well as carcass weights of elk *(Alces alces)* from the Hällefors territory in Sweden (90 individuals) were subjected to regression analyses. While the method was aimed at the elimination of environmental effects, the use of a wild animal species minimized the variability attributable to differences in the genetic background (i.e. breed), a constant source of bias in the osteological examination of sexual dimorphism in domesticates. As a result, sex-dependent changes in bone size, density and proportions could be clearly ascertained, and linked with the ontogeny of endocrine functions.

Keywords. Sexual dimorphism, allometric growth, European elk *(Alces alces)*.

Investigations related to the skeletal development of ruminants have largely been focused on the establishment of meat to bone proportions and size reconstruction. Bones of the so-called dry limb, that is the distal extremity segment, are widely used in predicting a variety of such traits. Both sheep and cattle metapodials are used to calculate withers height in archaeozoology (Boessneck, 1956; Matolcsi, 1970, etc.). Distal extremity portions as well as disarticulated tarsal and metapodial bones proved equally useful in the estimation of carcass characteristics of these two species (Palsson and Verges, 1952; Lőrincz and Lencsepeti, 1973; Noddle, 1973; Bartosiewicz, 1984*b*). Although sexual dimorphism has systematically been taken into consideration, many of these works are technical in nature and concentrate on the applied aspects of osteometrical research.

Similar studies of wild ruminants, especially Cervids include a number of species and a greater variety of skeletal parts (Lemppenau, 1964; Bosold, 1968; Chaix and Desse, 1981). Research into the age- and sex-dependent variability of dry limb bones in European elk carried out by Iregren (1975, 1985) is of particular importance to the results obtained in this paper which was aimed at the

quantification of the relationships between carcass weight and characteristics of the metacarpal ("canon bone") in the males and females of this species during postnatal ontogeny.

Materials and methods

The bone material for this analysis originates from central Sweden, where the hunting grounds of the Hällefors Sporting Club are located near the 60th degree of latitude (Iregren, 1975). All the animals studied here were killed during the 1971 hunting season. The 47 male and 43 female elks included both calves and adult individuals and form an ideal age sequence for the study of relative growth. Detailed information on the origin and age distribution of the sample have been published in the previously mentioned works by Dr. Iregren, 90% of whose material has been re-analyzed in this study. Here, allometric calculations have been applied to investigations of the growth of metacarpal bones relative to carcass weight. This method excluded the direct use of absolute age data resulting in the minimization of environmental effects which often act in a time-dependent fashion (for example seasonal changes in the availability of food). Thus, functional relationships between the parts of body may be more directly observed (Fábián, 1969).

Conforming to the traditional allometric method, decimal logarithms of the following variables were used in a linear regression analysis:

(1) The cubic root of dressed carcass weight was used in some cases as independent variable in the same way as is usual with live weight, a general descriptor of relative (biological) age. Carcass weight (Cw), however, was found to be better correlated with skeletal measurements than live weight in sheep (Palsson and Verges, 1952), probably as a result of excluding excess variability caused by individually different fat deposition and intestinal development. In the case of elk bulls, the presence or absence as well as size of antlers would also introduce a hardly controllable bias in the measurement of total live weight. The mathematical transformation was used to reduce the three-dimensional character of weight, thus rendering it more comparable with linear characteristics (Fábián, 1984 — personal communication). Otherwise the resulting allometric equations show regression coefficients that would have been reduced by two thirds. This decline of the slope indicates the much faster growth that appears when three dimensions are considered.

Of the dry limb bones available for study, metacarpals (Mc) were chosen as early fusing long bones having a sex-determined relationship with the rest of the skeleton (Bartosiewicz, 1985).

(2) The median length of metacarpals (Ml), used as independent variable, was measured along the median plane of the bone between the proximal end

and the corresponding point of the distal fusion line on the plantar side of the bone. This measurement is as an average 7.3% shorter than greatest length (Gl) in both sexes, but is highly correlated with that measurement, thus making the use of metacarpals from immature individuals (with unfused epiphyses) possible.

(3) Theoretical density of metacarpals (TD) was used both as an independent and as a dependent variable. During the measurement of metacarpals each bone was weighed (Bw). Since the weights of bones with and without epiphyses would not have been comparable, proximal breadth (Bp) and depth (Dp), smallest breadth (Sb) and smallest depth (Sd) of the diaphysis, as well as distal breadth (Db) and distal depth (Dd) measured at the lines of distal epiphyseal fusion were used to estimate the areas of cross sections at the three relevant metacarpal segments. Averaged areas of these "ellipses" (A_P, A_S, and A_D) were then multiplied by either greatest length or median length depending on the presence or absence of epiphysis. Dividing bone weight with the theoretical volume obtained this way a value, called theoretical density (TD), could be computed:

$$TD = Bw^{-1} [Gl; Ml(A_P + A_S + A_D) \cdot 3^{-1}]$$

Due to the hollow inside and concave wall structure of these tubular bones, the results systematically underestimate the actual, physical density of bone material, but are based on a number of important and widely used measurements (Duerst, 1926; von den Driesch, 1976) that make them perfectly suitable for the purposes of comparaison. They should, however, be regarded as strictly theoretical in nature.

(4) The slenderness index (Index) was plotted as dependent variable against theoretical bone density. It is usually based on the proportion between the smallest breadth of diaphysis and greatest length. In this case, however, median length (Ml) was divided by smallest breadth (Sb) to provide a number comparable even for young individuals which were killed before epiphyseal fusion could actually have taken place.

All these variables for each individual animal are listed in Tables I and II.

Allometric regression equations supported by high or moderately high correlations (Guilford, 1956), significant on a $P \leq 0.05$ level of probability, were required to accept the hypothesis that predictable curvilinear relationships between carcass weight and the previously described characteristics of the metacarpal bone exist. Should differences in the curves between female and male elks occur, they might cast light on further details of the skeletal development of ruminants.

Table I

Carcass weights and metacarpal measurements of female moose
(s and d stand for left and right side respectively)

Inventory no.	Cw kg	Bw g	Gl mm	Bp mm	Sb mm	Bd mm	Dp mm	Sd mm	Dd mm	Ml mm	TD g/cm³	Index
23 d	71	202		57.3	25.0	57.5	40.2	23.1	41.8	262.8	0.312	10.51
88 d	80	193		58.2	25.9	62.0	40.8	23.1	43.1	160.9	0.281	10.07
89 s	66	167		52.6	24.2	56.3	36.5	22.9	40.8	253.4	0.296	10.47
148 d	60	178		52.1	25.1	57.9	38.2	23.1	40.4	251.0	0.305	10.00
3 d	135	326		57.2	38.7	66.1	38.6	26.9	44.9	284.5	0.367	7.35
16 s	79	220		58.9	27.3	61.9	42.1	23.4	44.9	259.0	0.308	9.49
18 d	76	203		56.5	27.8	62.6	37.2	24.3	43.2	260.8	0.301	9.38
58 d	82	203		56.1	26.7	59.5	40.5	23.9	43.1	258.6	0.304	9.69
61 d	79	196		55.2	26.5	58.7	38.7	23.4	41.8	250.1	0.318	9.44
67 d	59	166		54.9	25.9	54.3	37.2	22.2	40.9	251.4	0.290	9.71
92 s	71	184		56.3	26.2	61.1	39.1	23.1	44.9	248.8	0.289	9.50
54 d	82	200		57.2	27.8	61.2	39.2	24.3	43.6	242.1	0.313	8.71
56 d	74	191		56.1	26.3	60.1	37.2	22.3	41.9	250.9	0.313	9.54
83 s	74	197		57.2	28.1	60.8	38.4	24.0	42.6	256.8	0.295	9.14
84 d	81	209		59.1	29.3	61.0	40.2	23.3	44.9	262.1	0.292	8.95
12 s	142	350		61.1	38.2	65.3	43.1	28.1	47.1	299.2	0.346	7.83
22 d	125	305		56.9	31.0	61.9	38.2	26.9	42.9	283.6	0.389	9.15
27 s	119	304		57.5	29.9	61.5	37.3	24.8	41.2	277.9	0.415	9.29
39 s	106	245		56.1	27.8	59.9	39.2	24.9	41.6	271.9	0.349	9.78
77 d	167	362		60.7	33.1	62.9	40.6	28.4	43.2	293.9	0.407	8.88
85 s	78	203		58.8	27.2	59.8	40.8	22.3	44.1	270.6	0.286	9.95
90 s	130	312		57.1	31.1	67.4	38.4	27.5	46.1	290.8	0.364	9.35
100 s	114	246		55.1	27.9	61.1	37.2	24.1	43.0	269.7	0.360	9.67
101 d	58	166		55.9	26.7	60.7	36.2	22.2	40.9	245.1	0.282	9.18
105 d	61	168		52.1	25.9	55.1	35.9	21.9	40.3	248.5	0.307	9.59
122 s	130	314		58.5	33.6	64.9	38.9	26.9	46.1	287.9	0.364	8.57
126 d	73	191		57.1	26.5	56.1	37.9	22.1	41.1	253.4	0.317	9.56
1 d	171	461	334.5	61.1	33.0	61.5	41.9	26.2	41.5	310.8	-0.464	9.42
2 d	195	461	331.2	61.9	33.2	61.3	43.7	27.8	40.5	308.1	0.454	9.28
5 d	211	334	312.6	60.2	33.7	60.1	41.9	26.9	38.2	289.9	0.395	8.60
6 d	144	531	348.2	62.5	37.2	63.2	43.7	28.1	40.8	325.0	0.468	8.74
8 s	219	501	341.2	58.1	36.2	65.7	41.8	25.7	39.5	318.5	0.479	8.80
14 s	172	447	331.3	56.7	35.4	61.9	37.9	27.9	40.3	308.1	0.471	8.70
17 d	248	614	349.2	61.6	39.2	68.5	44.2	29.8	42.9	322.8	0.500	8.23
20 d	131	444	330.3	59.1	33.5	62.7	42.2	27.0	42.7	307.1	0.445	9.17
24 d	263	543	343.2	62.8	36.1	67.8	43.6	28.8	41.3	319.6	0.472	8.85
28 d	228	505	334.1	61.5	35.1	62.3	41.9	28.4	40.7	315.9	0.488	9.00
52 d	200	456	345.4	61.8	33.5	63.6	40.5	28.2	41.1	321.9	0.467	9.61
63 d	213	532	322.4	58.9	36.2	60.6	40.9	27.9	39.2	304.1	0.553	8.40
75 d	184	453	337.8	61.9	32.1	60.0	42.1	28.3	39.2	278.2	0.456	8.67
87 d	215	411	312.1	57.1	33.5	59.4	39.2	26.1	37.1	292.1	0.483	8.72
97 d	224	507	331.0	62.9	34.8	62.8	44.1	26.2	40.8	310.6	0.486	8.93
108 s	207	518	348.4	63 9	36.1	64.1	41.3	28.1	40.7	324.9	0.466	9.00
110 s	219	528	344.0	63.8	35.8	65.2	42.4	28.7	43.1	320.4	0.467	8.95
119 s	188	305	320.0	62.0	30.2	61.1	42.8	25.2	38.0	299.4	0.335	9.91
125 d	173	414	312.9	55.6	33.2	58.9	40.0	26.2	38.1	291.8	0.486	8.79
xxx s	216	496	326.2	53.8	36.5	59.1	41.0	25.7	37.1	302.9	0.544	8.30

Table II

Carcass weights and metacarpal measurements of female moose
(s and d stand for left and right side respectively)

Inventory no.	Cw kg	Bw g	Gl mm	Bp mm	Sb mm	Bd mm	Dp mm	Sd mm	Dd mm	Ml mm	TD g/cm³	Index
29 d	55	162		49.2	32.1	50.3	33.6	20.7	36.8	238.9	0.361	10.34
50 s	65	170		52.1	24.7	56.1	37.1	21.0	39.2	247.2	0.314	10.01
51 s	61	163		51.9	24.2	55.3	36.1	20.9	40.7	242.9	0.313	10.04
103 d	63	169		50.4	23.5	56.0	35.2	21.1	39.1	242.9	0.334	10.34
124 d	70	163		51.2	23.1	52.6	34.8	21.1	39.7	246.6	0.328	10.68
64 s	109	291		58.6	28.2	57.4	39.2	26.2	40.9	262.2	0.145	9.30
25 d	88	196		52.8	27.2	55.7	35.0	23.1	41.0	254.3	0.340	9.35
55 s	71	172		52.6	25.3	57.2	35.4	21.8	40.6	242.4	0.320	9.58
65 d	63	173		54.8	26.0	57.9	36.7	22.1	39.9	245.1	0.305	9.43
72 d	70	195		55.2	27.1	58.1	37.2	22.5	40.9	257.1	0.317	9.49
81 d	48	167		53.2	25.8	54.1	36.7	22.1	40.2	237.2	0.317	9.19
116 d	70	188		54.1	27.5	59.6	37.9	22.8	41.7	248.4	0.308	9.03
53 s	61	165		49.1	26.3	57.5	34.9	22.1	40.9	249.1	0.301	9.47
76 d	70	195		55.5	27.0	57.1	36.1	22.8	41.0	247.7	0.335	9.17
95 d	68	175		54.2	27.9	58.4	37.1	22.1	40.1	243.6	0.300	8.73
19 s	54	160		51.7	24.9	54.1	36.9	22.0	38.8	236.1	0.313	9.48
26 d	65	180		51.4	25.1	57.2	36.8	21.8	41.0	243.1	0.330	9.69
86 d	69	201		54.6	27.2	59.2	37.2	23.1	43.5	250.3	0.327	9.20
99 s	68	180		53.2	26.1	58.0	36.1	22.2	41.4	251.9	0.310	9.65
117 d	77	196		54.8	27.3	57.2	37.2	22.9	41.9	259.0	0.316	9.49
149 d	76	201		53.4	27.2	56.1	36.6	24.1	40.2	252.9	0.340	9.30
16 d	150	396		54.9	32.5	61.2	39.1	25.2	36.2	312.2	0.477	9.61
13 s	164	384		58.9	35.2	63.2	40.8	27.9	45.1	288.9	0.430	8.21
21 d	124	211		55.1	29.1	57.9	36.1	25.8	40.3	283.1	0.301	9.73
68 d	163	323		58.3	30.9	61.2	39.5	26.1	41.8	293.0	0.397	9.48
69 d	132	292		56.5	30.0	59.9	37.2	25.9	39.9	278.2	0.405	9.27
85 s	78	203		58.8	27.2	59.8	40.8	22.3	44.1	270.6	0.286	9.95
93 d	167	361		58.2	33.1	62.0	39.2	28.6	42.7	293.8	0.421	8.88
113 d	135	284		54.6	30.8	55.2	38.1	24.1	39.4	271.2	0.423	8.81
4 d	167	369	331.1	57.9	30.6	63.8	38.6	22.9	38.6	310.8	0.416	10.16
7 d	136	414	332.8	58.6	34.1	59.2	38.1	26.2	38.1	309.9	0.453	9.09
57 d	198	405	334.8	55.9	31.5	58.6	38.2	27.2	40.2	314.2	0.454	9.97
59 s	153	365	313.1	58.6	32.1	61.2	41.1	25.5	37.1	292.4	0.417	9.11
68 s	163	409	332.9	55.1	31.2	58.1	40.7	26.1	38.1	311.2	0.463	9.97
60 s	171	411	324.5	56.2	33.1	58.9	39.4	26.1	40.0	301.8	0.463	9.11
66 s	148	396	330.1	57.8	31.1	63.2	40.4	25.2	39.1	305.7	0.430	9.83
74 d	157	417	329.2	58.2	32.4	57.3	39.2	27.5	38.0	308.7	0.468	9.53
82 s	142	299	297.5	50.0	28.4	53.8	36.1	23.6	37.1	278.1	0.453	9.79
94 s	142	420	336.8	60.3	34.1	61.1	41.2	29.2	42.1	312.6	0.411	9.17
107 s	204	504	346.9	62.1	35.2	64.1	41.1	29.1	44.8	323.1	0.454	9.18
118 d	144	336	319.0	56.1	31.7	57.2	38.0	25.8	36.5	298.8	0.412	9.43
120 d	228	440	317.9	56.7	33.9	58.9	38.1	24.9	37.1	296.2	0.520	8.74
123 d	184	469	323.9	57.1	36.1	59.3	39.8	27.9	37.8	302.7	0.506	8.39

Results

Mean values, standard deviations and coefficients of variation obtained by the conversion of univariate statistics of the allometric calculations are summarized in Table III. In spite of the relatively wide size range for individuals of varying ages, significant differences between the two sexes occurred in the case of carcass weight and median metacarpal length. Mean values of the theoretical density and slenderness index are less different due to their relatively large standard deviations as is shown by the coefficients of variation.

This quality of the variables is also mirrored in the explanatory power of allometric equations shown in Table IV. Highest coefficients of correlation were calculated between the decimal logarithms of the two variables expressed in absolute terms (carcass weight and median metacarpal length). The first two equations indicate a more degressive tendency of relative growth for

Table III

Univariate statistics obtained by the allometric equations

Variable/Group	Mean value	Standard deviation	Coefficient of variation
Carcass weight [Cw(kg)]			
males	124.177	1.622	0.013
females	102.569	1.585	0.015
Mc median length [Ml (mm)]			
males	289.499	1.327	0.005
females	272.273	1.355	0.005
Mc theoretical density [TD (g/cm)]			
males	0.378	1.236	3.270
females	0.364	1.255	3.448
Mc slenderness index (Index)			
males	9.141	1.072	0.117
females	9.436	1.056	0.112

Table IV

Allometric growth of the bone characteristics relative to carcass weight and theoretical density (For abbreviations and units see Tables I to III)

Groups	Allometric equation (intersection slope)	Coefficient of correlation
Males	lg Ml = 2.071 + 0.181 lg Cw	0.924***
Females	lg Ml = 2.011 + 0.210 lg Cw	0.943***
Males	lg TD = —1.275 + 0.407 lg Cw	0.916***
Females	lg TD = —1.119 + 0.338 lg Cw	0.681***
Males	lg Index = 0.888 — 0.057 lg TD	—0.529**
Females	lg Index = 0.953 — 0.016 lg TD	—0.206

metacarpal length in males than females. The allometric coefficients between carcass weight and theoretical density suggest that the relative increase of bone density is, on the other hand, more intensive in males. The least convincing allometric equations, relating the slenderness index to the theoretical density of metacarpals, show that the relative widening of the diaphysis follows the growth of bone density more consistently in males. In the case of females no significant correlation between these traits could be ascertained.

Discussion

The basic conclusions in this study have been drawn from the well-defined relationship between carcass weight and median metacarpal length. Graphic representation of the two equations is shown in Fig. 1 in natural units. As was evidenced by the small allometric coefficients, the growth of median metacarpal length relative to carcass weight may be described as a degressive, curvilinear relationship, which is more expressed in the case of male elk. Note, however, that the curves representing the two sexes are apparently parallel at the beginning of postnatal ontogeny. They intersect around the 140 kg threshold of carcass weight, when the relative longitudinal growth of metacarpals becomes much slower in males. At the same time, the curve remains close to linear for females. While a relatively small size difference may be found in terms of the metacarpal length between adult male and female individuals, mature carcass weight is much smaller in the case of females (just as their live weight is approximately 70% of that of a bull elk). The same phenomenon is shown in Fig. 2. While the age distribution of female and male elk in the sample is largely symmetric (Iregren, 1975: Table I), this tendency may only be observed at lower carcass weights ($Cw < 100$ kg). Adult elk bulls in the sample have evidently larger carcasses than those of females, while sexual dimorphism in the median length of metacarpals is usually far less pronounced or even statistically insignificant (Chaix and Desse, 1981).

Epiphyseal fusion, that is the cessation of longitudinal bone growth in general, appears when the sex hormones reach a critical level in the blood of subadult individuals (Arey, 1965). It is well known, however, that distally located extremity bones fuse earlier than most parts of the skeleton. The fusion of metacarpals occurred between one and two years of age in the population under study here (Iregren, 1975), which is long before sexual maturation is actually completed (elk calves, usually born between the end of May and middle of June in the area under discussion may mate by the fall of their third year of life, although practically no young bulls have a chance during the first few rutting seasons). The early ossification of metapodials means that circulating factors related to sexual maturation do not effect the length of metacarpals as much as the growth of other long bones or musculature.

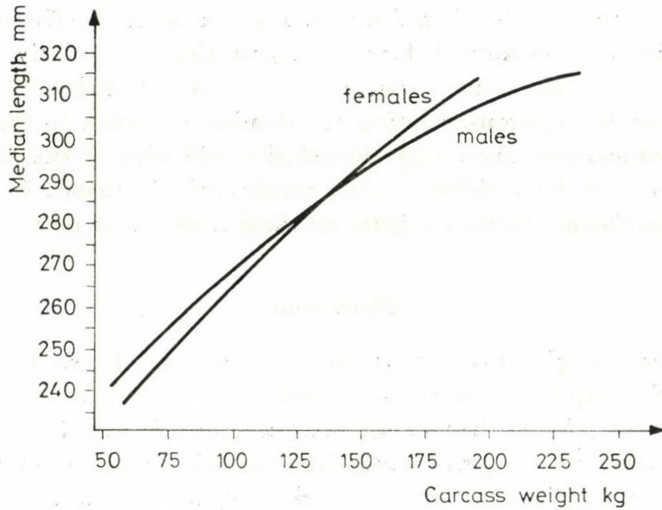

Fig. 1

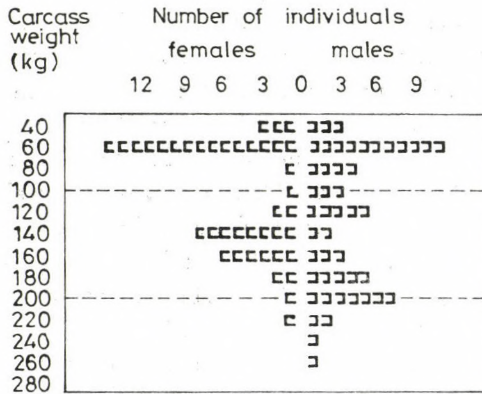

Fig. 2

Growth and sexual maturity depend on many synthetic processes and most of the hormones are involved in their control. Gonadal hormones influence the development of secondary sexual characteristics such as size in both sexes. Of these, androgens are synthesized in the Leydig interstitial cells of testes, and their secretion is regulated by the production of ICSH in the adenohypophysis. This process already begins in male elk calves and is first accelerated in yearlings. As is shown by the negative evidence of castration in domestic animals, the otherwise normal synergism between testosterone and growth hormones is damaged by gonadectomy in males, inducing relative hypothyreosis slowing down intermediary metabolism. The lack of testosterone thus indirectly is related to a lower intensity of oxygene absorption by the

tissues. At the same time, increased glyconeogenesis (in capons, for example: Bökönyi and Bartosiewicz, 1983) exacerbates protein catabolism and the protein content of the bone matrix decreases delaying the process of epiphyseal fusion. Consequently, skeletal growth and muscular development are retarded.

Although the previously mentioned phenomena occur in default of testosterone, they illustrate the role played by male gonadal hormones in stimulating the increase of musculature at an age when epiphyseal fusion has already limited the longitudinal growth of metapodials. The example of elk is an illustration of the differential effect of sexual maturation on two types of tissue. It contributes to the observations made on various cattle long bones which also display a sexual dimorphism more or less proportional to the degree of their exposure to gonadal hormones. In the case of a well defined population of wild animals, however, even smaller samples were reliable, providing more clear-cut results than a genetically heterogeneous set of cattle skeletons used in a previous study of sexual dimorphism in skeletal development (Bartosiewicz, 1984*a*).

The practical implication of this paper is that in the case of sufficiently homogeneous game populations even relatively small samples may be used in the sex dependent prediction of carcass weight on the basis of metacarpals. Although the occurrence of elk has only been sporadic in Hungary (Topál and Vörös, 1984), the method in general may enhance the *in situ* estimation of meat yield from large bodied Cervids. Field work of wildlife management officials may also be facilitated when reconstruction of meat loss due to natural causes such as predator activity or epidemics is studied on the basis of offal. Last but not least, the allometric equations may be used for size estimation in archaeozoology when complete elk metacarpals are brought to light during the course of excavations. These practical applications are made possible by the high coefficient of correlation calculated between carcass weight and median length of the metacarpal which ensures that estimates obtained by the M1 = = f(Cw) function would largely agree with those calculated from Cw = f(M1). Detailed study of this latter relationship, however, is beyond the focus of this paper concerned with basic research.

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OCCURRENCE OF TWO NEW *GOUSSIA* SPECIES IN THE INTESTINE OF THE STERLET *(ACIPENSER RUTHENUS)*

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Two new *Goussia* species, *G. vargai* and *G. acipenseris*, are reported from the intestine of sterlets *(Acipenser ruthenus)* caught in the River Danube. The oocysts of both parasites are exreted in unsporulated state; in tap water at 20 °C they sporulate within 48 h. By histological methods the oocysts and developmental stages of the parasites can be demonstrated from the mucosal epithelium of the pyloric appendages and small intestine. *G. vargai* develops in a usual location, i.e. in the cytoplasm of epithelial cells, whereas *G. acipenseris* can be found in epiplasmal location, under the cell membrane of epithelial cells, protruding into the intestial lumen.

Keywords. *Goussia vargai, G. acipenseris,* coccidium, sterlet *(Acipenser ruthenus)*, intestine, development, location.

In recent years the demonstration of fish-parasitic coccidia has become successful. In 1983, Dyková and Lom reported 127 species belonging to the genera *Cryptosporidium*, *Crystallospora*, *Eimeria*, *Epieimeria*, and *Goussia;* since then their number has increased by further 10 species described by Li and Desser (1985*a*, *b*), Landsberg and Paperna (1985) and Jastrzebski (1982, 1985). In spite of this, no coccidia have been known to occur in species belonging to the Acipenseridae family.

In the present paper, two new *Goussia* species are reported from the intestine of the sterlet *(Acipenser ruthenus)*.

Materials and methods

The sterlets examined were mature, several years old fish that had been caught from the reach of the Danube near Paks (Hungary) and transported, for propagation, to a fry-hatching pond farm early in May. In 1984 and 1985, 16 and 24, respectively, of the sterlets were subjected to parasitological examination; their intestines were examined for the presence of coccidia. The intestines of the fish, kept on nets or in basins for a few days, contained no remnants of feed. The mucus covering the intestinal epithelium could be lifted off easily. The intestine was always divided into three parts; the first contained the glandular body including the pyloric appendages, the second the foregut, whereas the third the hindgut.

The mucus and mucosal scrapings taken from the different parts of the intestine were examined under the microscope, and the oocysts found in them were made to sporulate in tap water, in small petri dishes, together with the mucus. Bacterial growth was prevented by adding penicillin and streptomycin.

The intestines of some of the fish were processed histologically as well; the intestinal portions were fixed in Bouin's solution, embedded in paraffin, and the sections were stained with haematoxylin and eosin.

Results

Fourteen of the 16 fish examined in 1984 and 21 of the 24 ones examined in 1985 had coccidium oocysts in their intestine. In the mucus taken from the intestinal wall, unsporulated oocysts of two different sizes and round shape were found. The oocysts were of granular structure and had a uniformly dark appearance (Fig. 1). The smaller oocysts were 7 to 8 μm, while the larger ones 11 to 14.5 μm, in size. In tap water, at 20 °C, sporulation was completed within 24 h, the oocysts increased in size and transformed into *Goussia*-type coccidia. After 48 h the sporocyst residuum completely filled the sporocysts and after 72 h it became compact. Based upon their size and characteristic location determined by histological methods, the oocysts proved to belong to two hitherto unknown *Goussia* species, whose description is as follows.

Goussia vargai n. sp. (Fig. 2)

(The parasite was named in honour of Dr. István Varga, the noted Hungarian coccidiologist.)

Host and locality: *Acipenser ruthenus*, River Danube, around Paks (Hungary).

Location: mucous membrane of the intestine and pyloric appendage.

Hosts/infection: 40/35.

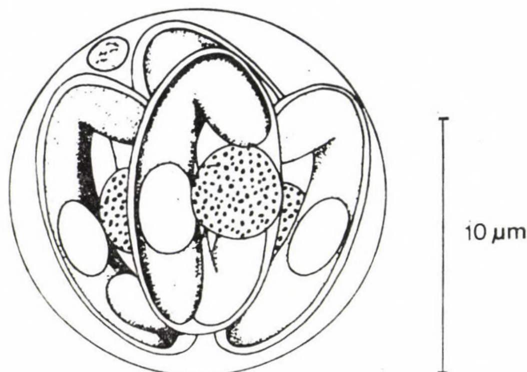

Fig. 2. G. vargai oocyst

Description (on the basis of 25 oocysts examined): The oocysts are spherical or short-ellipsoidal in shape. In sporulated state, the spherical oocysts are 12.6 to 20 μm (average: 15.88 $\pm$ 1.84), whereas the short-ellipsoidal ones 15–20 $\times$ 14–19 μm (average: 18.06 $\pm$ 1.3 $\times$ 16.38 $\pm$ 0.98) in diameter. The oocysts have a thin, colourless, single-layered wall; within them the sporocysts show a relatively loose arrangement. There is neither oocyst residue nor micropyle, but there exist one or two amorphous polar granules 1 to 1.5 μm in size. The sporocysts are elongated ellipsoidal in shape (Fig. 3) and 10.5 to 14 $\times$ 4.5 to 6 μm (11.6 $\pm$ 0.64 $\times$ 5.1 $\pm$ 0.76) in size. The sporocysts are composed of two hemispheres and have a very thin wall; in the oocyst they are arranged irregularly but mostly in one direction. In the sporocyst there are two vermiform sporozoites arranged in head to tail presentation, with one end reflexed. Without the reflexed end, the sporozoites are 9 to 11.5 μm (10.4 $\pm$ 1.69) in length and 1.7 to 2.2 μm (1.85 $\pm$ 0.09) in thickness. In the sporozoites there is a large refractile globule. After 72-h sporulation the sporocyst residue is 3 $\times$ 4 μm in size, compact, and short-ellipsoidal in shape.

The oocyst is excreted from the fish in unsporulated state. The intestine of severely infected fish contains merozoites 9.5 $\times$ 2.2 μm in size.

Histological studies. The developing oocyst and early developmental stages of *G. vargai* were found in the epithelial cells of pyloric sacs of the glandular body and in those of the foregut mucosa. The meronts (Fig. 4), each containing 16 merozoites 11 $\times$ 2 μm in size, were located in the cytoplasm of the epithelial cells, and varied between 9–11 $\times$ 11–13 μm in size. On one occassion I found a subepithelially-located meront 23 $\times$ 17 μm in size; it contained 40 merozoites 8 $\times$ 2 μm in size (Fig. 5). The macrogamonts (Fig. 6), sized 7–13 $\times$ 12–17 μm, and the microgamonts (Fig. 7) 10–13 $\times$ 15–17 μm in size were mostly in the cytoplasm between the nucleus of epithelial cells and the lumen; occasionally, however, they were demonstrable basal to the nucleus (Fig. 8). The degree of infection varied between fish, but within a given fish there was no difference

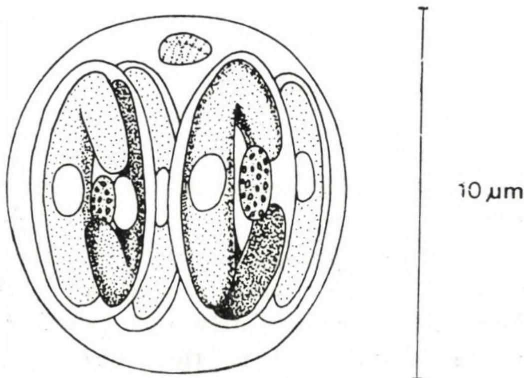

Fig. 9. G. acipenseris oocyst

between the number of parasites present in the foregut and in the pyloric sac.

Goussia acipenseris n. sp. (Fig. 9)

Host and locality: *Acipenser ruthenus*, River Danube, around Paks (Hungary).

Location: mucous membrane of the intestine and pyloric appendage; the part of epithelial cells immediately under the surface.

Hosts/infection: 40/28.

Description (on the basis of 25 oocysts examined): the oocysts (Fig. 3) are short-ellipsoidal in shape and 9.6–10.7 × 7.5–9.7 μm (average: 10.17 ± ± 0.54 × 8.47 ± 0.55) in size; their wall is very thin, single-layered and colourless. The sporocysts show rather compact, usually unilateral, arrangement within the oocysts. There is neither oocyst residue nor micropyle, but there exists a polar granule 0.5 to 1.0 μm in size. The sporocysts are elongated ellipsoidal and 6.6–8.8 × 3.0–4.3 μm (average: 8.11 ± 0.83 × 3.4 ± 0.36) in size. The sporocyst consists of two hemispheres and has a very thin wall. In the sporocyst there are two vermiform sporozoites arranged in head-to-tail presentation with one end reflexed. Without the reflexed end, the length of the sporozoites is 7.2 to 8 μm (average: 7.72 ± 0.17), while their thickness is 1.1 to 1.6 (1.31 to 0.18) μm. The sporozoites contain a large refractile globule. After 72-h sporulation the short-ellipsoidal sporocyst residue is 2.5 × 1.3 μm in size.

The parasite is excreted from the fish in the form of unsporulated oocysts.

Histological examination. Both the developing oocysts and the early developmental stages of *G. acipenseris* can be found in characteristic location, i.e. in epiplasmal position, under the luminal cell membrane of the mucosal epithelial cells lining the glandular body's pyloric sacs and the intestine, and protruding from the cells (Fig. 10). The 4–6 × 4.5–7 μm-sized meronts (Fig. 11) contain 8 merozoites 3 × 2 μm in size. The macrogamonts (Fig. 12) are 6.5–8 × 8.9, while the microgamonts (Fig. 13) 4–5.5 × 6.5–7.5 μm in size.

Discussion

The results indicate that in the intestine of the sterlet and other acipenserids severe coccidial infection, similar to that found in other fish species, may develop. In the spring period studied, most of the sterlets prepared for spawning were infected by two types of oocysts. The larger oocysts occurred somewhat more frequently. The structure of both kinds of oocysts was of *Goussia*-type, i.e. the wall of the sporocysts was composed of two equal halves joining one another in the middle by sutures; thus, the sporocysts were morphologically typical of the genus *Goussia* Labbé, 1896, revalidated by Dyková and

Lom (1981). On the other hand, the histological examinations revealed fundamental differences in the development of the two species. *G. vargai*, like the majority of species belonging to the Eimeriidae family, developed in the cytoplasm of epithelial cells of the intestine and pyloric appendages, whereas *G. acipenseris* occupied an epiplasmal position in the very same epithelial cells. Development similar to that of the latter species was first reported by Léger and Hollande (1922) and Léger and Bory (1932), who thought that the eel parasite *Eimeria anguillae* and *E. pigra* parasitizing the rudd developed extracellularly, attached to the surface of the epithelium. Dyková and Lom (1981) took into account the same postulated epicellular development when they established the genus *Epieimeria*, into which, however, they were unable to assign *Goussia (Eimeria) pigra*, a species having Goussia-type oocysts. Recently, using electron-microscopic methods, Molnár and Baska (1986) have demonstrated that *Epieimeria anguillae* does not have extracellular merogony and gamogony as these stages develop within the epithelial cell but in a parasitophorous vacuole bordered solely by the cell membrane. In the present case no electron-microscopic studies have been performed; however, it still seems doubtless that *G. acipenseris* has a mode of development similar to that of *E. anguillae*. Since *G. pigra* and *G. acipenseris* cannot be classified into the genus *Epieimeria*, the justification of attempts made by Dyková and Lom (1981) and Levine (1983, 1984) at dividing the genus *Eimeria*, formerly considered to be an integral whole, is questionable. At the same time, our studies, together with the results of Landberg and Paperna (1985) and Jastrzebski (personal communication) call attention to the fact that the epiplasmic mode of development and excretion of oocysts from the fish intestine in unsporulated state may be much more frequent than indicated by data of the contemporary special literature.

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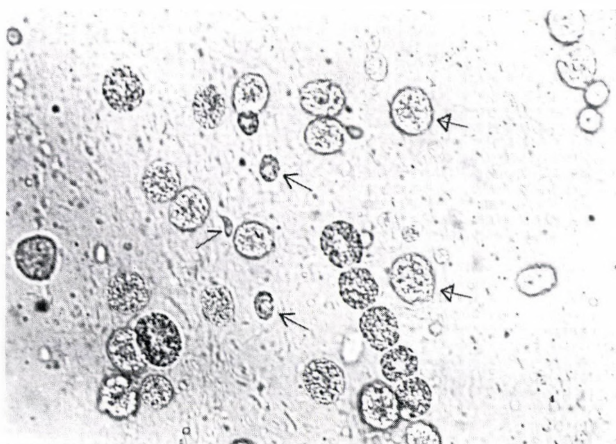


Fig. 1. Unsporulated oocysts in the intestine of sterlet. ↑: *G. vargai* oocysts; ↑: *G. acipenseris* oocysts; ↑: merozoite. Native preparation, × 700

Fig. 3. Sporulated oocysts. ↑: *G. vargai* cysts after sporulation for 48 h; ↑: *G. vargai* sporocysts after sporulation for 72 h; ↑: *G. acipenseris* oocyst. Native preparation, × 1500

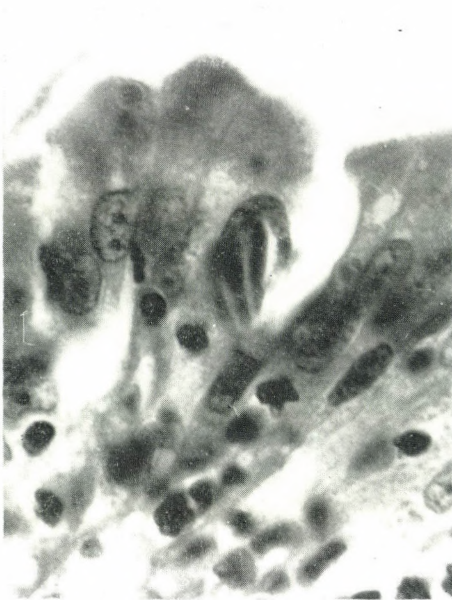


Fig. 4. G. vargai merozoites in an epithelial cell. Haematoxylin and eosin, × 1500

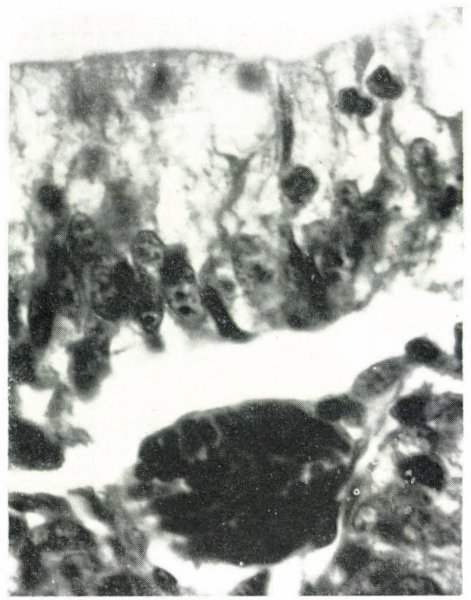
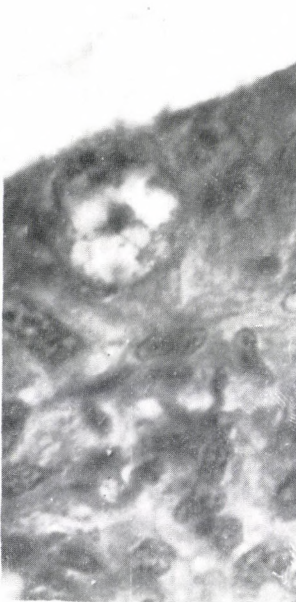
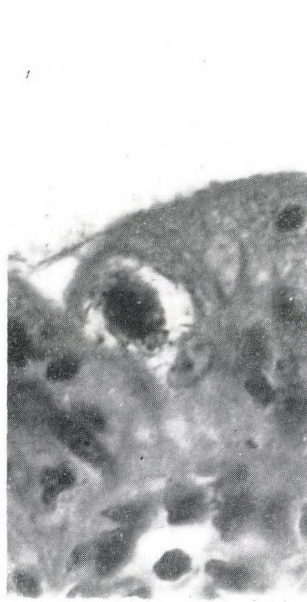


Fig. 5. G. vargai meront deep in the epithelium. Haematoxylin and eosin, × 1500





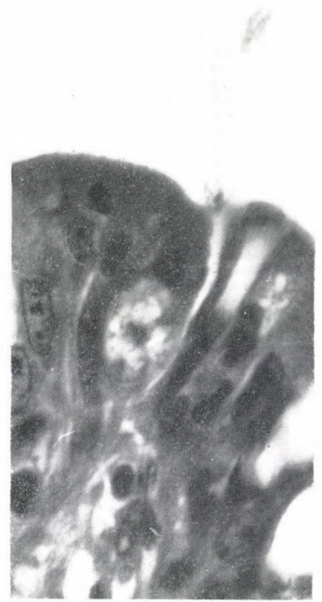


Fig. 6. G. vargai macrogamont in an epithelial cell. Haematoxylin and eosin, × 1500
Fig. 7. G. vargai microgamont in an epithelial cell. Haematoxylin and eosin, × 1500
Fig. 8. G. vargai macrogamont, situated basal to the nucleus of the epithelial cell. Haematoxylin and eosin, × 1500

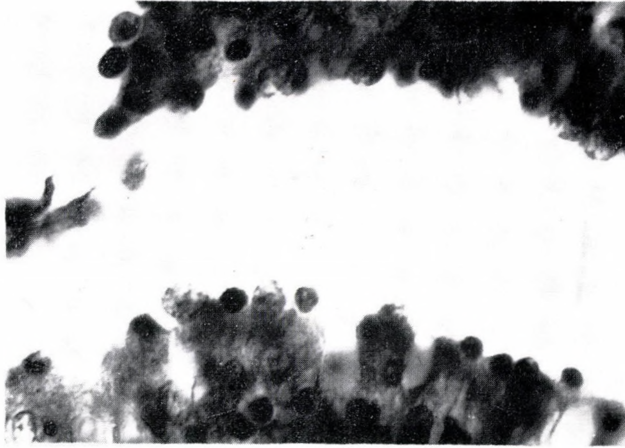


Fig. 10. G. acipenseris trophozoites in epiplasmal position. Haematoxylin and eosin, × 1500

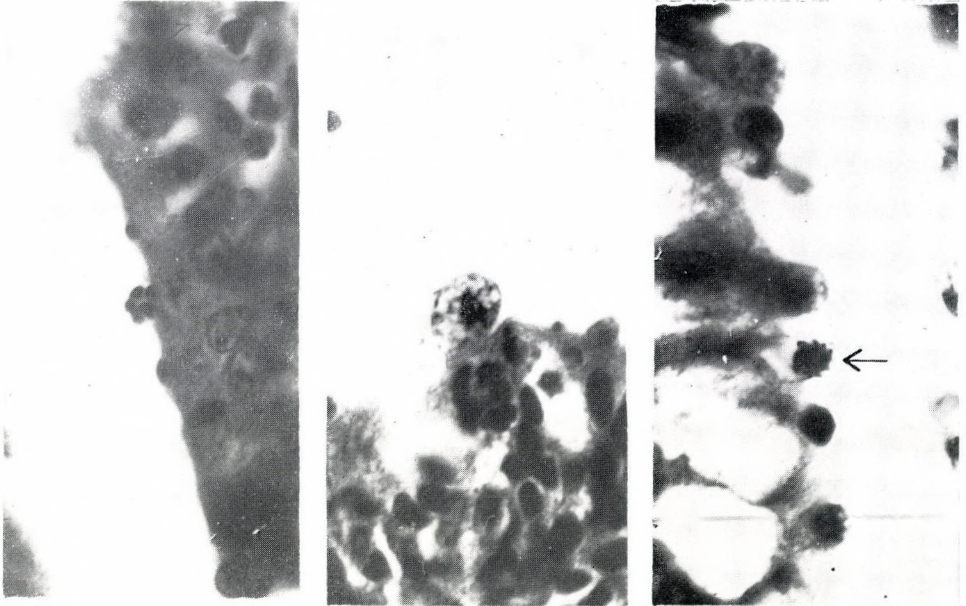


Fig. 11. G. acipenseris meront, apparently in extracellular position. Haematoxylin and eosin, × 1500

Fig. 12. G. acipenseris macrogamont. Haematoxylin and eosin, × 1500

Fig. 13. G. acipenseris microgamont (↑). Haematoxylin and eosin, × 1500

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STUDY OF THE POSTULATED IDENTITY OF *HOFERELLUS CYPRINI* (DOFLEIN, 1898) AND *MITRASPORA CYPRINI* FUJITA, 1912

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In the kidneys of two- and three-summer old common carp (*Cyprinus carpio* L.) obtained from pond farms the so-called *Hoferellus cyprini* nodes were found to occur mainly in the autumn, plasmodia regarded as *Mitraspora cyprini* in the winter, whereas spores early in the spring. It is concluded that the species *Mitraspora cyprini* Fujita is synonymous with *Hoferellus cyprini* Doflein. *H. cyprini* develops in a one-year cycle: from October its early vegetative stages are situated intracellularly in the epithelium of renal tubules, parasitize several neighbouring epithelial cells, and thus constitute a single parasitic focus. From December the parasites gradually get into the lumen of renal tubules, in which they develop into plasmodia. Spores may develop already in the tubules; however, in most cases spore formation takes place in the ureter or urinary bladder.

A redescription of spores of *Hoferellus cyprini*, whose validity was contested by several authors, has also been performed.

Keywords. *Hoferellus cyprini* (Doflein, 1898), *Mitraspora cyprini* Fujita, 1912, identity, common carp (*Cyprinus carpio* L.), spore, morphology, description, developmental cycle.

Hoferellus cyprini was described from common carp (*Cyprinus carpio* L.) by Doflein (1898), who found in the renal tubules of common carp amoeboids 20 to 30 μm in size and pyramidal, longitudinally striated spores having characteristic polar projections. The polar capsules lay in the sutural plane. Other *Hoferellus* spp. were found in *Carassius auratus gibelio* by Achmerov (1960) and in crucian carp *(Carassius carassius)* by Golikowa (1960); they described these as *H. carassii* and *H. schulmani*, respectively. The spores of these parasites resembled those of *H. cyprini*, with the difference that there were bristle-like filaments at their ends.

The development of *H. cyprini* was studied by Plehn (1924), who, in addition to plasmodia and spores parasitizing the lumen of the renal tubules, found the early stages developing in the form of intracellular foci in the epithelial cells of the renal tubules. She established that the parasite was characterized by a one-year developmental cycle which was completed by the end of winter.

In Japan, from the renal tubules of common carp and goldfish Fujita (1912) reported a myxosporean resembling *H. cyprini*. The parasite was identical in size with *H. cyprini*; however, at the ends of its spores there were bristle-

like filaments and the suture connecting the two hemispheres of the spores ran in the plane between the spore capsules. Fujita (1912) named this parasite *Mitraspora cyprini.*

Thorough investigations into the development and pathology of *M. cyprini* were made in Japan by Ahmed (1973*a* and *b*) who attributed the disease characterized by kidney enlargement in the fish to this parasite. During the one-year developmental cycle of the parasite, in the summer and autumn Ahmed (1973*b*) found intracellular stages in the epithelium of renal tubules, in the winter coelozoic plasmodia in the renal tubules, whereas in the spring spores in the lumen of the renal tubules.

After its description *H. cyprini* was not reported to occur in Europe over a long period, and its existence was indicated by curricular data only (Schäperclaus, 1954; Bauer et al., 1969). Its recurrence was reported only in 1981 by Lom and Dyková. It was also Lom and Dyková (1981) who demonstrated the first European occurrence of *M. cyprini*, in Czechoslovakia. Subsequently Körting and Hermanns (1984, 1985) demonstrated both *H. cyprini* and *M. cyprini* from ponds of Southern Saxony. Furthermore, from the illustrations made by Lom and Dyková (1981) it undoubtedly appeared that the formation described in Hungary by Molnár (1980) as a *Myxobolus* cyst was actually a *Hoferellus* stage.

In the present paper, by comparing data of the literature with results of our own investigations, we furnish evidence that *M. cyprini* is a synonym of *H. cyprini.*

Materials and methods

The test material consisted of two- and three-summer old common carp, originating from various pond farms and submitted in 1982 and 1983 to the Central Veterinary Institute for routine examinations, primarily for demonstrating *Sphaerospora renicola* infection.

In 1984 and 1985 our studies were restricted mainly to the Fish Farm of Lovászpatona, where three generations were kept together in a pond and where *Hoferellus* infection far exceeded the average in Hungary. To survey *Hoferellus* infection, two- and three-summer common carp from this pond were examined at two-week intervals in the autumn and spring, and at one-month intervals in the winter. In the summer only common carp fry were submitted to the laboratory. Three to ten fish were dissected on each occasion. The kidneys were removed in their entirety, in association with the ureter and urinary bladder, and one part of each organ was studied in fresh.

From all the kidney parts examined, impession smears were prepared and stained with Giemsa; deep-frozen sections were made from one fish on each

occasion. Kidney pieces of fish that had proved positive in the fresh examination were fixed in 10% formalin or Bouin's solution, embedded in paraffin, and sections 4 to 8 μm in thickness prepared from them were stained with haematoxylin and eosin and according to Farkas and Mallory.

Results

Our studies were prompted by two observations made during routine diagnostic work. Studying the development of *Sphaerospora renicola* and the infection caused by it, we regularly found the *Hoferellus* foci (Fig. 1) described by Plehn (1924) in the kidneys of two- and three-summer old common carp. At other times plasmodia of an unknown myxozoan (Fig. 2) were detected in the ureters; they proved to be stages of *Mitraspora cyprini* only after a thorough investigation and, particularly, after that spores had developed from them by the spring. *Hoferellus* foci were found to occur primarily in the autumn and early in the winter, while *Mitraspora* plasmodia in the winter and spring.

Systematic studies were conducted to clarify the relationships existing between these observations.

Hoferellus foci were demonstrable at earliest in fish examined in October. At that time foci described by Plehn (1924) were found in the kidneys of about one-quarter of the two- and three-summer fish submitted to the laboratory from different fish farms of Hungary, while in about two-thirds of those kept under regular control in the Fish Farm of Lovászpatona. These foci were 100–120 × 120–200 μm in size and spherical or elongated in shape; they were located along the renal tubule and imitated its shape. Histologically, intensive protozoan infection of the epithelium of renal tubules was established in certain circumscribed areas of the kidneys. The myxosporidian developmental stages, 6 to 8 μm in diameter and granular in structure, filled up and enlarged the epithelial cells and pushed the cells' nucleus towards the basement membrane. The borders of the cells were not distinguishable by light microscopy. In their cytoplasm the large parasitic mass got in the immediate vicinity of the lumen of renal tubules; however, it was still separated from the latter by a narrow cytoplasmic zone on which the brush border was well visible. Occasionally, part of the cells surrounding the lumen remained free from infection (Fig. 3).

In December, in a part of the foci the tubular epithelium showed discontinuities, and *Hoferellus* stages 6 to 8 μm in diameter appeared in the lumen of the tubules. From December onwards *Hoferellus* foci in the kidney gradually decreased in number, and in March such foci were demonstrable only exceptionally.

At the time when the number of *Hoferellus* foci started to decrease (in December and occasionally already at the end of November) oval or amorphous

myxosporean plasmodia 15–40×15–30 μm in size occurred in the ureter; later on they gradually increased in number. These plasmodia were found freely in the lumen or, occasionally, attached loosely to the wall of the ureter. In intensive infection the plasmodia filled part of the renal tubules as well (Fig. 4). In March and April the plasmodia got in more and more distal portions of the ureter and spores developed within them. Spore formation took place primarily in the ureter and urinary bladder; however, in intensive infection numerous spores were found also in the convoluted tubules. Three to ten spores were formed within each plasmodium. In fresh preparations the spores, 9×6.6 μm in size on the average, had the shape described by Hofer (1898), as shown in Fig. 5; however, a more thorough examination demonstrated the presence of caudal filaments and striae on them. The suture, which was hard to visualize, was found to run in the plane between the two spore capsules.

Based upon the developmental stages examined and the study of 25 spores, we give a redescription of the parasite as follows.

Hoferellus cyprini (Doflein, 1898)

The vegetative stages fall into two types. The early developmental stages are spherical trophozoites 6 to 8 μm in diameter (Fig. 6); they develop intracellularly in the epithelium of the renal tubules and contain 7 to 8 daughter cells (Fig. 7). These stages develop in a given portion of the tubules and from spherical or oval foci 100–200×120–200 μm in size in the kidneys. The late vegetative stages can be found in the lumina of renal tubules and ureters: these are transparent plasmodia of irregular shape and 15–40×15–30 μm in size. The endoplasm of plasmodia has granular structure and contains large refractile droplets. The plasmodia are polysporoblastic and 3 to 10 spores are formed within them. The mature spores (Fig. 8) are short ellipsoidal, 8.5 to 10 (9.0) μm long, 5.2 to 7.1 (6.6) μm wide and 5.2 to 5.8 (5.6) μm thick. Their anterior end is rounded and the posterior one is flattened. The polar capsules lay in the sutural plane. The suture only slightly protrudes over the surface of the spore and is hard to observe. The surface of the spore shell is furrowed; on it run 20 longitudinal striations which start from the apex of the anterior end and, slightly deviating from the spore shell, form a characteristic rim around it. As a continuation of the ribs, twenty bristles 4.5 to 5 μm in length project caudally from the spore. The spores contain two equal-sized polar capsules 3.5 to 4.2 (3.8) μm in length and 2.1 to 2.2 (2.15) μm in width; their anterior end is tapered, and the polar filament has four or five convolutions in them.

These spores, identified by us with spores of *H. cyprini*, essentially correspond to those described by Fujita (1912) and Kudo (1920) as *M. cyprini*, and differ from those illustrated by Ahmed (1973*a*) only in their somewhat "stubbier" conformation.

Discussion

From the present studies it may be concluded that the species descriptions of Doflein (1898) and Fujita (1912) cloak the same parasite; i.e. the species *Mitraspora cyprini* Fujita, 1912 is a synonym of *Hoferellus cyprini* (Doflein, 1898). Over a long period researchers were prevented from recognizing their identity because the species and genus description of Doflein (1898) was accepted by the subsequent investigators (Plehn, 1924; Achmerow, 1960; Golikowa, 1960; Shulman, 1966) practically without any revision. These investigators failed to notice that Doflein had illustrated the course of sutures of *H. cyprini* spores incorrectly. Doflein (1898) must have imagined the hardly-observable sutures of *H. cyprini* to resemble those of *Myxobolus* species, with which he was very familiar. Plehn (1924) studied mainly the development of *H. cyprini* and only little did she deal with its spore morphology. On the other hand, Golikowa (1960) and Achmerov (1960) noticed that there were long filaments at the ends of *Hoferellus* spores; however, they, too, illustrated the plane of sutures erroneously. At the same time, Achmerov (1960) was right reporting that at the posterior half of the approximately oval spores there was a ring-shaped formation.

Fujita's (1912) representation of *Mitraspora cyprini* reported by him from common carp and goldfish was essentially correct, and his description was accepted by Kudo (1920) and Ahmed (1973*a*).

With regard to the fact that, despite the erroneous representation, Doflein's (1898) description enjoys priority, the species described and the genus created by Fujita (1912) should be considered synonyms of the species *Hoferellus cyprini* and the genus *Hoferellus* Berg, 1898, respectively.

In addition to the morphological similarity of the spores, also the developmental cycle and the intracellular stages are strongly suggestive of the identity of *M. cyprini* with *H. cyprini*. Both Plehn (1924) and Ahmed (1973*a*) found that the parasite followed a one-year cycle, its early developmental stages appeared in the autumn, the plasmodia in the winter, while their spores in the renal tubules appeared in the spring. No difference exists between *M. cyprini* and *H. cyprini* in the morphology of the vegetative stages either, since the renal tubule lesions reported from goldfish by Ahmed (1973*a*) and Hoffmann (1984) as mitrasporosis were identical with those reported by Plehn (1924), Lom and Dyková (1981) and Körting (1984), and also with our findings.

The development of *H. cyprini*, as revealed by the present studies, is essentially consistent with that suggested by Ahmed (1973*a*), with the difference that we, due to the low incidence and intensity of infection, had no opportunity to study the early developmental stages occurring in the summer. The developmental stages found by us correspond to those described by Plehn (1924); however, Plehn (1924) assumed that the *Hoferellus* organisms released from the

epithelium of the renal tubules into the lumen formed spores immediately. Contrarily, the present findings indicate that the *Hoferellus* developmental stages released into the lumen first form a multinucleated plasmodium there, and spores appear only after a developmental period of some weeks or months.

Our observations contradict the hypothesis of Dyková et al. (1983) suggesting that intracellular *Hoferellus* stages are some other myxozoan developmental stages that have got to a deadlock of development. We can imagine that after the disruption of the foci some of these stages get into the interstices and are destroyed there; however, most of the developmental stages undoubtedly get into the lumen and form mature spores. Furthermore, we presume that the intracellular stages do not always form large, cyst-like foci; sometimes they infect solitary cells as well. Such parasites, considered by Lom and Dyková (1981) to represent *Sphaerospora renicola* developmental stages, can also be identified, in all probability, with *H. cyprini*.

Shulman (1966) considered the genus *Mitraspora* to be a synonym of the genus *Sphaerospora* Thelohan, 1982; thus, the identity of the genus *Hoferellus* with sphaerospores might be suggested. However, such an identity is excluded by the considerable development-biological differences existing between the two groups of parasites, despite their possible morphological resemblances.

Further studies are needed to clarify whether the very same *Hoferellus* species parasitizes the common carp, goldfish, crucian carp and *Carassius auratus gibelio*, and whether the species described by Achmerov (1960) and Golikowa (1960) should be regarded as synonyms of *H. cyprini* or as one or more distinct species.

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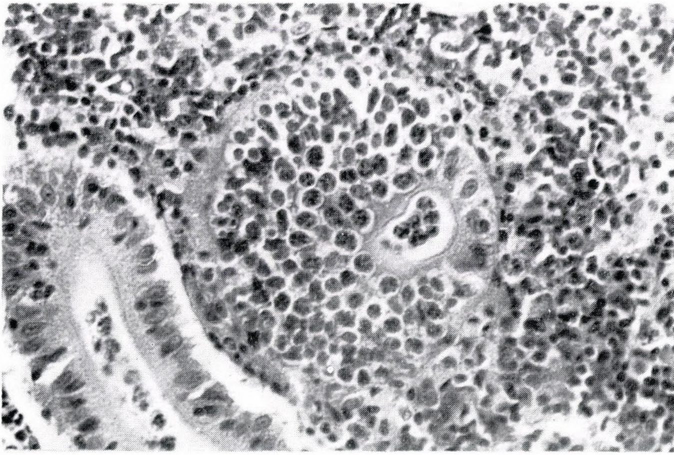

Fig. 1. Hoferellus "nodule" around the renal tubule in common carp. *Hoferellus* developmental stages are situated within the epithelial cells. In the lumen of the renal tubules developmental stages of *Sphaerospora renicola*, occurring as a frequent concomitant parasite, can be seen. Haematoxylin and eosin (H–E.), × 500

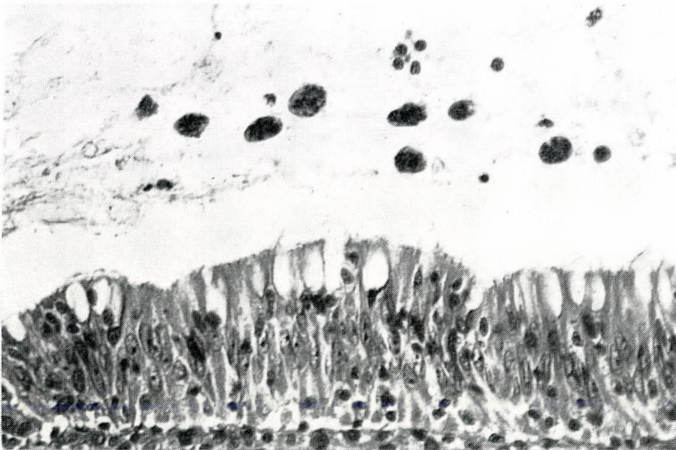

Fig. 2. Hoferellus plasmodia in the lumen of the ureter. H–E., × 600

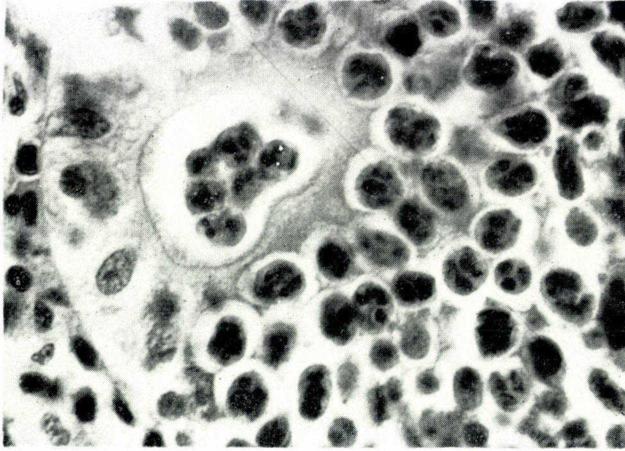

Fig. 3. Magnification of a part of Fig. 1. Intracellular *Hoferellus cyprini* trophozoites exceed in size the sphaerospores occurring in the lumen. On one side of the tubule there still are intact epithelial cells. *Hoferellus* trophozoites filling the infected epithelial cells are separated from the lumen of the tubule by a narrow cytoplasm. H–E., × 1200

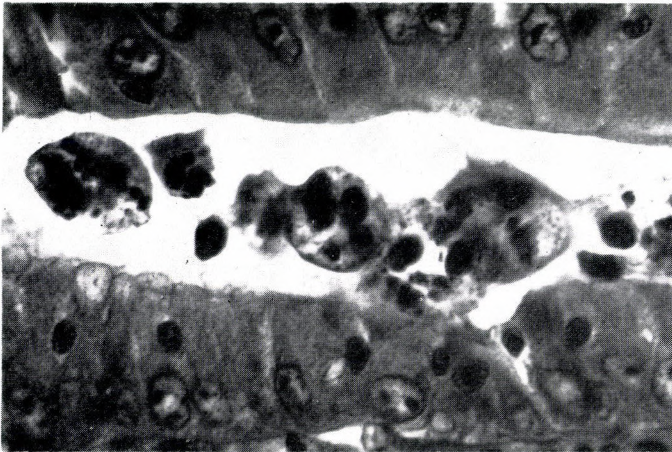

Fig. 4. Spore-containing *Hoferellus* plasmodia in the lumen of a renal tubule. H–E., × 1800

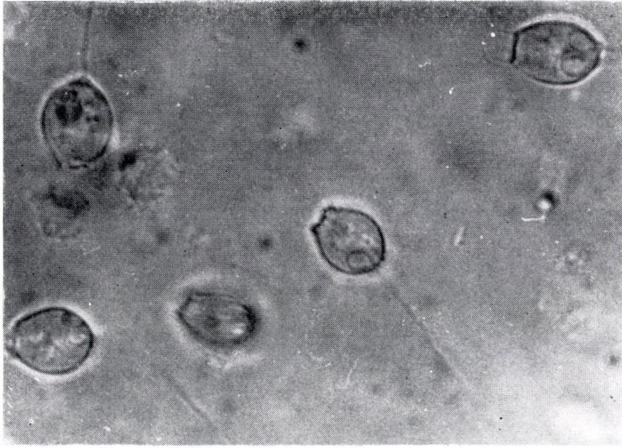

Fig. 5. Hoferellus cyprini spores. In part of the spores the filaments have been ejected from the polar capsule. Fresh preparation, × 2000

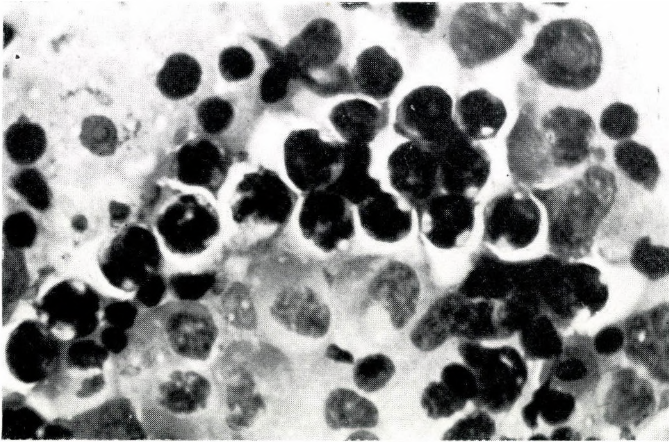

Fig. 6. Spherical trophozoites obtained from a *Hoferellus* nodule. Impression smear stained with Giemsa, × 1800

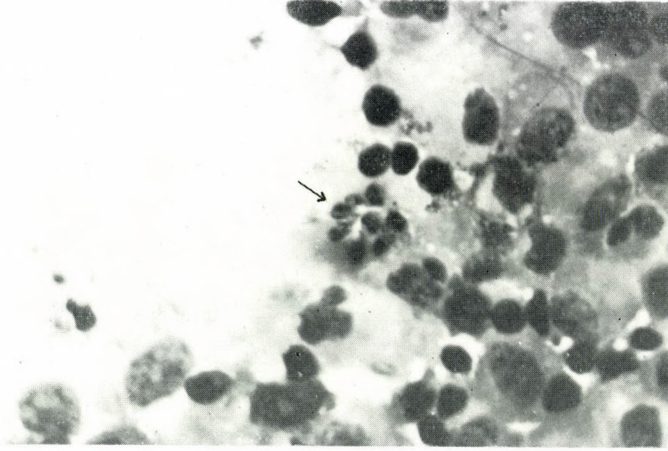

Fig. 7. Squashed *Hoferellus* trophozoite (arrow), consisting of secondary formations. Impression smear stained with Giemsa, × 1800

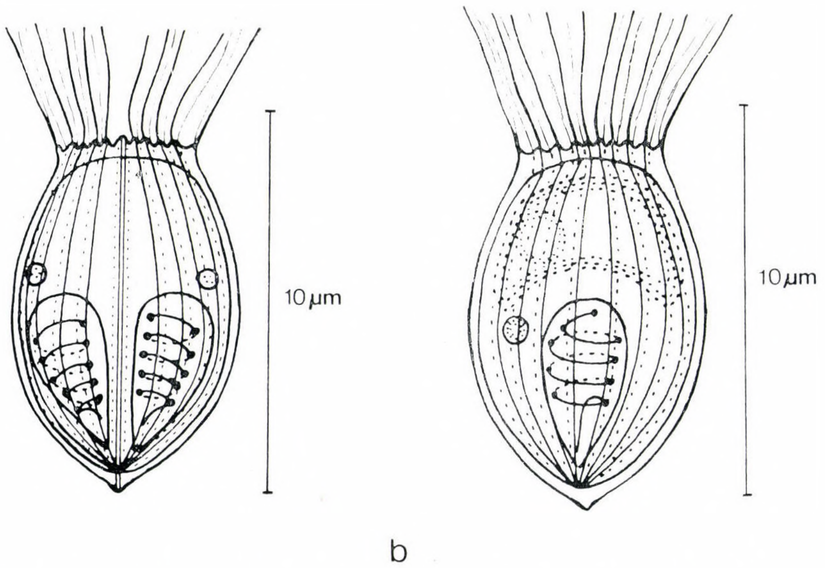

Fig. 8. Schematic presentation of *Hoferellus cyprini* spores. *a:* spore in frontal view; *b:* spore in transversal view

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INCORPORATION OF CARBON FROM ^{14}C-LABELLED PRECURSORS INTO MAJOR CHEMICAL FRACTIONS OF *HAEMONCHUS CONTORTUS* IN VITRO

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Adult *Haemonchus contortus* (Nematoda: Trichostrongylidae) has the potential of synthesizing proteins, nucleic acids and carbohydrates from simple precursors viz., acetate, glucose, palmitic acid and carbon dioxide. Out of the precursors employed, palmitic acid is the most efficient precursor of proteins, glucose of nucleic acids and acetate of carbohydrates. Incorporation of carbon from acetate and HCO_3^- into nucleic acids was insignificant.

Incorporation of carbon into proteins and carbohydrates gives an evidence for the operation of the tricarboxylic acid cycle. Further, synthesis of nucleic acids is indicative of the operation of the pentose phosphate pathway. Possible mechanisms for the involvement of the various precursors in the biosynthesis of these chemical fractions are discussed.

Keywords. *Haemonchus contortus*, proteins, nucleic acids, carbohydrates, acetate, glucose, palmitic acid, CO_2.

Haemonchus contortus (Nematoda: Trichostrongylidae) is one of the most common pathogenic nematodes, found parasitic in the abomasum of sheep, goats, cattle and other ruminants. The carbohydrate and lipid metabolism of the adult parasite has been studied extensively (Ward, 1974; Kaur and Sood, 1982, 1983; and Kapur and Sood, 1984*a*, 1986).

However, in *H. contortus* no report has been available regarding the metabolism (biosynthesis and catabolism) of other metabolites viz. proteins and nucleic acids, though amino acid biosynthesis has been reported (Kapur and Sood, 1984*b*). Also, there is no evidence of glycogenesis. Therefore, in the present studies, an attempt has been made to investigate whether or not it has the potential of biosynthesizing these components. Further, the relative importance of the different precursors has also been evaluated.

Materials and methods

Collection of parasites. Adults of *H. contortus* of both sexes were recovered from the abomasum of goats *(Capra hircus)*, procured from local abattoirs. The specimens were thoroughly washed in saline to remove the adhering materials.

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Incubation medium. A modified Tyrode's solution was used (Ward, 1974).

Incubations. Four batches of worms in numbers corresponding to 0.5 g each were transferred to flasks containing 10 ml of Tyrode's solution. To each flask 12.5 μCi of the ^{14}C-labelled precursor was added. The precursors employed included U-^{14}C-D-glucose, sodium-1-^{14}C-acetate, sodium-^{14}C-bicarbonate and ^{14}C-palmitic acid. These were incubated at 39 $\pm$ 1 °C for 4 h on a metabolic shaker. After the incubation period, a few drops of chloroform: methanol (2 : 1 v/v) were added in order to stop the incorporation. Media were poured off and the worms washed with distilled water several times.

Extraction. Worms were homogenized in 10 per cent trichloroacetic acid (TCA) for 4 min and centrifuged for 10 min at 4 °C with 1800 $\times g$. The precipitate was further treated to separate its protein and nucleic acid components. This was achieved by extraction of the precipitate with 5% TCA at 0 °C. It was then heated for 10 min and centrifuged for 10 min at 4 °C and 1800 $\times g$. Proteins were recovered in the precipitate and nucleic acids in the supernatant.

The supernatant of the first extraction was extracted with chloroform: methanol (2 : 1 v/v) in order to separate carbohydrates from the lipids.

Measurement of radioactivity. In order to measure radioactivity, aliquots of proteins, nucleic acids and carbohydrates were taken into scintillation vials. The scintillation fluid had the following composition: 4 g 2,5-diphenyloxazole (PPO), 200 mg 1,4-bis-2-(5-phenyloxazolyl-benzene (POPOP), 20 ml ethylene glycol, 100 ml methanol, 60 g naphthalene and the volume made to one litre with dioxan. Radioactivity was measured in Beckman-7000 β-scintillation spectrometer.

Results

Results of the incorporation studies into major biochemical fraction of adult *H. contortus* are given in Table I.

It is evident that adult *H. contortus* incorporated carbon into proteins, nucleic acids and carbohydrates from all the precursors employed. In the case of proteins, there was maximum incorporation of carbon from palmitic acid and minimum from HCO^-. (There was no significant difference in the incorporation from acetate and glucose.)

For nucleic acids, glucose and palmitic acid are efficient precursors, whereas incorporation from acetate and HCO_3^- is insignificant. For carbohydrates, incorporation was in the following order: acetate $>$ glucose $>$ HCO_3^- $>$ palmitic acid. (There was no significant difference in the incorporation from acetate and glucose.)

Table I

Incorporation of ^{14}C into the proteins, nucleic acids and glucose + glycogen fractions of adult *H. contortus*

Chemical fraction	Precursor			
	Acetate	Glucose	Palmitic acid	Bicarbonate
Proteins	4.382 (0.337)	5.196 (0.080)	8.184 (1.747)	2.634 (0.229)
Nucleic acids	1.685 (0.129)	8.211 (0.127)	7.444 (1.588)	1.264 (0.109)
Glucose + glycogen	8.574 (0.660)	8.116 (0.126)	2.396 (0.511)	4.089 (0.354)

The results are expressed as dpm$\times 10^{-4}$/g fresh weight. Figures in parentheses indicate nmoles incorporated into g fresh weight.

Discussion

Parasitic helminths are capable of rapid protein synthesis, and incorporation of labelled amino acids into proteins has been demonstrated in a wide variety of helminths (Barrett, 1981). In the present studies, *H. contortus* has been demonstrated to be capable of synthesizing protein. The scheme proposed previously for the involvement of these precursors into amino acids in *H. contortus* (Kapur and Sood, 1984*b*) also holds true for the protein synthesis because ^{14}C is first incorporated into amino acids, which are then assembled into proteins. And this is indicative of the operation of the tricarboxylic acid cycle. Also low activities of aconitase and isocitrate dehydrogenase in adult *H. contortus* (Kaur and Sood, 1983) lend the support that the tricarboxylic acid cycle has only a minor function as regards energy production. It is possibly concerned with the interconversions of carbon-skeletons into amino acids (Saz and Vidrine, 1959; Oya et al. 1962, 1965) and hence proteins.

Incorporation of glucose carbon into proteins has been demonstrated in a number of parasitic nematodes viz. *Cooperia punctata, Dirofilaria immitis* and *Ancylostoma caninum* (Perez-Gimenez et al., 1967; Jaffe and Doremus, 1970; Slonka et al., 1973). Presently, a considerable amount of label from palmitic acid has been detected in proteins of *H. contortus.* This is well in agreement with the findings on *A. caninum* and *C. punctata* (Perez-Gimenez et al., 1967; Slonka et al., 1973). Incorporation of acetate and CO_2 into protein is being reported for the first time in a parasitic nematode, though incorporation into amino acids has already been well established (Rothstein, 1965; Rothstein and Tomlinson, 1961). Incorporation of CO_2 into proteins might be explained by implicating succinate as the primary product of CO_2 fixation (Saz and Vidrine, 1959), as in *Ascaris.*

In *H. contortus* incorporation from CO_2 and acetate is negligible in comparison to that from glucose and palmitic acid. Nucleic acid biosynthesis

from these precursors has been reported only in a few species viz., *Ascaris, C. punctata, Litomosoides carinii* (Entner and Gonzales, 1959; Slonka et al., 1973; Akinwande and Akinrimisi, 1980). It has been suggested that glucose is partially decomposed by the hexose monophosphate pathway into ribose and hence incorporated into RNA. Thus, the present studies give us an evidence for the operation of the pentose phosphate pathway in *H. contortus;* key enzymes viz., glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase of this pathway have already been demonstrated (Kaur and Sood, 1984).

Glycogen is the chief energy reserve in parasitic nematodes (Von Brand, 1979). Thus, synthesis of carbohydrates is probably the most important anabolic process in these parasites. Glycogenesis from glucose has been reported in several species (Wang and Saz, 1974). In the present studies, glycogenesis in *H. contortus* has been demonstrated from all the precursors employed. Similarly, it has been reported in other helminth species viz. *Hymenolepis diminuta* from CO_2 (Prescott and Campbell, 1965), *Ascaris, A. caninum,* and *C. punctata* from palmitic acid (Fernando and Wong, 1962; Perez-Gimenez et al., 1967; Sanhueja et al., 1968; Greichus and Greichus, 1970; Slonka et al., 1973). Incorporation of acetate carbon into glycogen, reported in the present studies, had also been demonstrated in *H. diminuta* (Jacobsen and Fairbairn, 1967).

However, in *H. diminuta* no incorporation of fatty acid carbon into glycogen has been reported (Jacobsen and Fairbairn, 1967). Biosynthesis of glycogenic amino acids from these precursors in *H. contortus* (Kapur and Sood, 1984*b*) could also account for glycogenesis reported in the present studies. Also, there is a postulation of glyoxylate cycle (Kornberg and Krebs, 1957) being the pathway involved in incorporation of fatty acid carbon into glycogen. So far this cycle has been demonstrated to be operative only in *Ascaris* egg (Barrett et al., 1970). In *H. contortus,* propionate has been found to be one of the end products of glucose metabolism (Ward, 1974). Thus, it is possible that propionate by means of CO_2 fixation is converted to succinate, which is glycogenic. And in the present studies, glycogenesis has been reported from CO_2. Also, it has been demonstrated that CO_2 is required for optimum synthesis of glycogen (Fairbairn et al., 1961).

It is concluded that adult *H. contortus* is capable of synthesizing proteins, nucleic acids and carbohydrates from simple precursors in vitro. These studies are indicative of the operation of the tricarboxylic acid cycle and the pentose phosphate pathway in the parasite. However, further detailed studies need to be carried out before any definitive conclusion can be drawn. Thus, possible pathways involved in the biosynthesis of these metabolites need to be explored.

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PATHOLOGICAL AND IMMUNOLOGICAL STUDIES ON CHICKEN EMBRYOS AND DAY-OLD CHICKS EXPERIMENTALLY INFECTED WITH *MYCOPLASMA GALLISEPTICUM*

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Embryonated hen's eggs obtained from an SPF flock were inoculated with *Mycoplasma gallisepticum* into the yolk sac on the 7th or 14th day of incubation, and SPF day-old chicks into the airsacs or intranasally. Subsequently, the growth rate of the chicks, the gross and histopathological lesions, the success rate of attempts at re-isolating the pathogenic agent, and the appearance of humoral and cellular immune responses were monitored.

The chicken embryos inoculated into the yolk sac exhibited retarded growth, an acute serous, then subacute necrotic, inflammation of the chorioallantoic membrane (CAM), heterophil-granulocytic interstitial hepatitis, generalized heterophil-granulocytic perivasculitis and acute, then subacute, bronchopneumonia. In the chicks inoculated into the airsacs or intranasally, lympho-histiocytic rhinitis and multiple focal pleuritis as well as airsacculitis accompanied by lymphocytic and histiocytic infiltration were found.

Lymphocytes segregated from the peripheral blood recognized the antigen already when the chicks were 10 days old. This ability tended to increase up to 30 days of age. The antibody titres remained low throughout.

Keywords. *Mycoplasma gallisepticum*, chicken embryo, day-old chick, pathology.

Mycoplasma gallisepticum is transmitted via the egg. *M. gallisepticum* alone can severely affect the chicken embryo, primarily before immunocompetence develops. In chicken embryos inoculated on the 7th day of incubation, inflammation of the chorioallantoic membrane (CAM), bronchopneumonia, pericarditis, inflammation of the nasolacrimal duct, arthritis, and perivascular infiltration by heterophilic granulocytes all over the body were observed (Goto et al., 1984).

In chicks, *M. gallisepticum*-induced lesions include lymphocytic and histiocytic tracheitis, pneumonia and airsacculitis as well as arthritis and synovitis (Kirklyn and Olson, 1967; Grimes and Rosenfeld, 1972).

Ultrastructurally, mycoplasmas can be observed both on the surface of the infected mucosal epithelial cells, among the microvilli, and intracellularly (Boam and Sanger, 1970).

Following experimental infection of 4 days old, 7 weeks old and 20 weeks old chickens with *M. gallisepticum*, specific antibodies demonstrable by the

haemagglutination-inhibition (HI) and slide and tube agglutination tests appeared after 2 weeks (Roberts et al., 1967; Adler et al., 1973; Timms and Cullen, 1975).

In the present experiment chicken embryos were inoculated with *M. gallisepticum* in the first and second thirds of incubation, and chicks immediately after hatching, to obtain data on the pathology, immunology and diagnosis of embryonic and hatchery infections.

Materials and methods

Experimental chicken embryos and day-old chicks

Two hundred Leghorn chicken embryos and 120 chicks obtained from a controlled SPF flock were used. The eggs were incubated in a RAGUS-60 type laboratory incubator. The embryos were assigned into 6 (A, P_A, K_A, B, P_B, K_B), whereas the chicks into 4, groups (C, D, P_C and K_{CD}). The groups and treatments are shown in Table I. At three-day intervals, three viable embryos from each group were processed for bacteriological and histopathological examination, and some of their organs for ultrastructural study. The embryos found dead at the candling performed daily were examined by microbiological and histological methods. Embryos of groups A and B were monitored for mycoplasma, while in groups P_A, K_A, P_B and K_B only the dead embryos were usedfor mycoplasma isolation. Samples for bacteriological and histopathological examination were taken from 3 of the chicks hatched from embryos of groups B, P_B and K_B each, and from 3 chicks of groups C, D, P_C and K_{CD} each, on the 1st, 5th, 10th, 20th and 30th days after hatching, when certain organs of group B, C, D and K_{CD} chicks were used for mycoplasma isolation, while their blood serum was examined in the HI test. The lymphocyte-stimulation and immunorosette-formation tests were done with blood samples of 5 chicks each of group C and P_C, on the 1st, 10th and 20th days after hatching.

In evaluating the results of our morphological and immunological examinations, the data obtained with SPF birds earlier (Glávits et al., 1981) were used as comparison, in addition to the noninoculated control birds included in the present study.

Histological examination

After opening their abdominal and thoracic cavity, the embryos were fixed in 5% formalin (pH 7.2) buffered with NaH_2PO_4 and NaOH, then, after removing their limbs and head, the embryos were embedded in paraffin. Sections were cut in three different planes (in the plane of the vertebral column and in two other planes parallel with it). The CAM of the embryos and the spleen,

liver, pancreas, kidneys, brain, lungs, trachea, nasal conchae, heart, bursa of Fabricius, thymus and tarsal joint of the chicks were fixed as above and embedded in paraffin (the tarsal joint was previously decalcified). The sections were stained with haematoxylin and eosin. For a more detailed study of certain leucocytes, Giemsa stain and the periodic acid–Schiff (PAS) reaction combined with diastase digestion were used.

Electron microscopy

Tissue samples were taken from the CAM, liver and lungs of 3 embryos per group and from the liver, lungs, nasal conchae and kidneys of 3 chicks per group, from areas selected on the basis of the light-microscopic results. The samples were processed and examined as described earlier (Glávits, 1981).

Microbiological examination

Strain S_6 of *M. gallisepticum* was used. The strain was grown in BEG medium (Ernø and Stipkovits, 1973). The inoculum was 100 μl of 24-h broth cultures; inoculation was carried out as described in Table I. Re-isolation of mycoplasmas was attempted from the yolk sac of embryos that were killed, or died, during the experiment and from the heart blood of day-old chicks, on a solid medium (medium B). In addition, the nasal mucosa, trachea and airsac

Table I

Groups and treatments

Group	Time of treatment	Mode and dose of infection
"A": 50 eggs	day 7 of incubation	100 μl of *M. gallisepticum* broth culture, into the yolk sac; 2×10^6 CFU/ml
P_A: 25 eggs	day 7 of incubation	100 μl broth culture into the yolk sac
K_A: 25 eggs	—	—
"B": 50 eggs	day 14 of incubation	100 μl of *M. gallisepticum* broth culture, into the yolk sac; 2×10^7 CFU/ml
P_B: 25 eggs	day 14 of incubation	100 μl broth culture, into the yolk sac
K_B: 25 eggs	—	—
"C": 50 chicks	day 1 after hatching	100 μl of *M. gallisepticum* broth culture into the airsac; 3×10 CFU/ml
"D": 30 chicks	day 1 after hatching	One drop of *M. gallisepticum* broth culture into the left and right nostril each, 3×10^7 CFU/ml
P_C: 20 chicks	day 1 after hatching	100 μl broth culture, into the airsac
K_{CD}: 20 chicks	—	—

wall samples of the day-old chicks were inoculated into BEG broth. Culturing was performed as described by Stipkovits et al. (1977). The possible presence of other bacteria was checked by smearing organ samples on blood agar.

Immunological studies

The specific antibodies present in the serum were demonstrated by the haemagglutination-inhibition (HI) test, using 4 HA units of the 24-h broth culture of *M. gallisepticum* strain S_6.

For the lymphocyte-stimulation test, lymphocytes were separated from 2-ml blood samples with Ficoll-Paque, by centrifuging the samples at 20 °C with 1000 *rpm* for 30 min. Subsequently the lymphocytes were resuspended in Hanks' solution enriched with 5% fetal calf serum. Cell density was adjusted to 10^5/ml. The cell suspension obtained from each bird was distributed in 4 Leighton tubes (0.2 ml/tube). The test was carried out as described by Szent-Iványi et al. (1981). For the immunorosette-formation test, to the cell suspension obtained from the individual birds an erythrocyte suspension (density: 10^6/ml) treated or not treated with 0.4 ml mycoplasma antigen (1 mg/ml) was added which had been pretreated with 1 : 125,000 tannic acid.

Statistical evaluation

The mortality rates and incidence of lesions in the various groups were compared by the χ^2 test, while the growth rate of embryos and chicks of the different groups by Student's one-sample *t* test.

Results

Microbiological examinations

The results of the re-isolation attempts are summarized in Table II. Mycoplasma was isolated from 98% and 72% of group A and group B embryos, respectively. No mycoplasmas were isolated from the placebo-treated and control embryos that died during incubation. Four embryos were found to be contaminated bacteriologically; these were excluded from the evaluation. From the airsacs of hatched chicks of group B no mycoplasmas were isolated; however, mycoplasma isolation was successful from the heart blood of a bird killed on the 5th day. In group C, the airsacs and heart blood of one chick yielded mycoplasma. In group D, mycoplasma was demonstrated in the nasal cavity of 91.7%, whereas in the airsac and trachea samples of 58.3% of the chicks. The control chicks (groups K_{CD} and P_C) remained free from infection throughout the 30 days of the experiment.

Table II

Re-isolation from embryos and day-old chicks of *Mycoplasma gallisepticum* used for infection

	Embryos						Chicks			
	A	P_A[4]	K_A[4]	B	P_B[4]	K_B[4]	B[1]	C[1]	D[2]	K_{CD}[3]
Tested	48	3	1	25	1	1	12	12	12	12
Positive	47	0	0	18	0	0	1	1	11	0

Remarks: [1]re-isolation was made from the heart blood, airsacs and trachea; [2]re-isolation was made from the trachea and nasal cavity; [3]samples were taken only from the airsacs, trachea and nasal cavity; [4]only the embryos that died during incubation were tested

Pathological study

The average body size of *group A* embryos, as determined on the basis of body length measured in the plane of the vertebral column, was inferior to that of the control embryos of group P_A from PI (post-inoculation) day 3 up to the time of hatch-out (Fig. 1). By the one-sample *t* test no significant difference ($P > 0.1\%$) was found between the means of data measured on the different days. Deaths due to infection started to occur, first sporadically, around PI day 3; at hatching time the number of unhatched embryos was outstandingly high. The incidence of deaths is given, in a cumulative manner, in Fig. 2. The χ^2 test revealed that in this group the proportion of total embryonic losses was significantly higher than the sporadic deaths observed in group P_A ($P < 0.01$) and group K_A ($P < 0.001$); however, it did not differ significantly from the incidence of deaths found for group B ($P > 0.05$). In this group only two non-viable embryos hatched. The incidence of lesions is summarized in Table III.

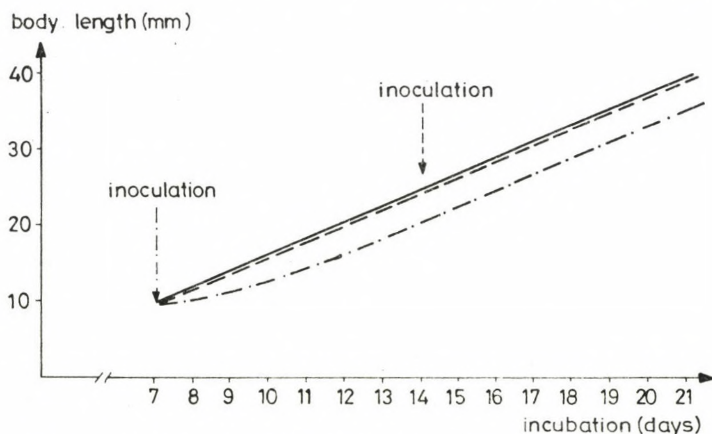

Fig. 1. Body length of chicken embryos infected with *Mycoplasma gallisepticum* (continuous line: control; dotted line: embryos infected on day 7 of incubation; broken line: embryos infected on day 14 of incubation)

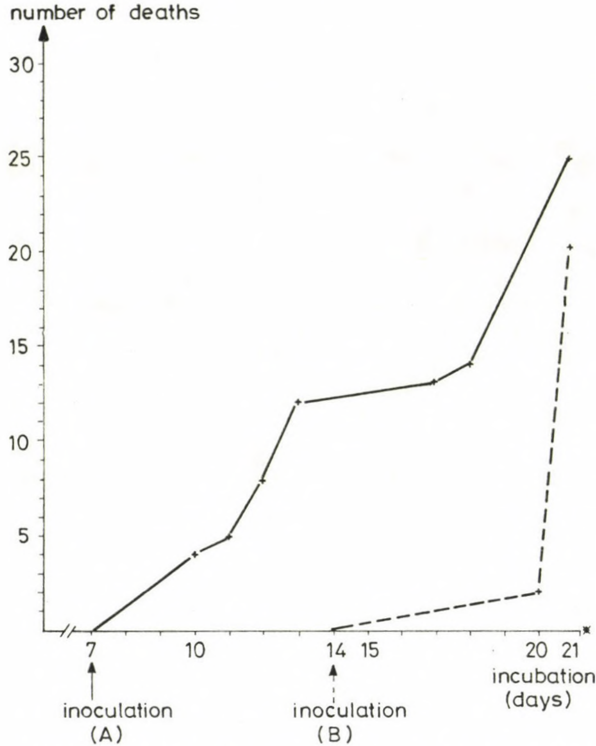

Fig. 2. Cumulative mortality of chicken embryos infected with *Mycoplasma gallisepticum* (*deaths on day 21 of incubation represent unhatched embryos)

As compared to the controls, in embryos killed or succumbed on PI day 3 the CAM was somewhat thickened, oedematous and, here and there, it exhibited small haemorrhages. Histologically, within the slightly dilated blood vessels the heterophils proliferated and, extravasating into the mesenchyma, infiltrated the perivascular areas. In the epithelial layer of the CAM the cells were swollen, their cytoplasms underwent vacuolar degeneration, or the epithelial cells even detached. Large numbers of heterophils were seen in the sinusoids of the liver and in the tissues surrounding the major blood vessels, whereas in other organs hyperaemia and, in some places, minor diapedeses occurred.

The CAM of embryos killed on PI days 6, 9 or 12, or of those succumbed prior to hatching, was markedly thicker than that of the controls, and contained extensive greyish-yellow, non-detachable areas. The embryos' organs showed a slight yellowish discolouration. Histologically, on the surface of the CAM there were large necrotic areas extending into the mesenchyma; these areas were demarcated by infiltrating inflammatory cells, mainly heterophils (Fig. 3). Here and there in the necrotized tissues and on their surface there was a fibrinous exudate.

The periportal tissues of the liver exhibited an expressed, mainly heterophil-granulocytic, infiltration consisting of 5 to 20 rows of cells (Fig. 4). By PI day 12, mononuclear cells (lymphoblasts, lymphocytes, histiocytes and plasma cells) had appeared among the infiltrating cells. The hepatocytes underwent a pathological simple, or in some places a necrobiotic, fatty infiltration, with the appearance of bilirubin cylinders in Disse's spaces situated between the rows of hepatocytes.

Heterophil-granulocytic infiltration of the blood vessel walls and perivascular areas was demonstrable also in organs other than the liver (e.g. spleen, kidneys, adrenals, lungs, pancreas, muscular layer of the gizzard, etc.) and even in the skeletal muscles and embryonic mesenchyma.

In the lungs of the succumbed embryos, in the lumen of the developing parabronchi there was an infiltration consisting of detached and degenerated epithelial cells, serum, fibrin and granulocytes. On PI days 12–14 in these infiltrations the necrosis and condensation of cellular elements and, around them, signs indicative of an inflammatory demarcation (acute or subacute bronchopneumonia) containing foreign-body giant cells were observed (Fig. 5). In this group the inflammation of the CAM, heterophil-granulocytic hepatitis, bronchopneumonia and heterophil-granulocytic infiltration of the blood vessel walls occurred significantly more frequently than in the group inoculated with medium P_A. All lesions except the heterophil-granulocytic infiltration of the blood vessel walls were of significantly higher frequency in group A than in group B (Table III).

The colonization of bursal follicles by lymphocytes was less expressed in group A than in the control group. The organs of hatched and subsequently killed chicks exhibited the lesions described above, although the lung lesions were circumscribed and mild.

The average body size of *group B* embryos, measured as the body length of embryos split in the plane of the vertebral column, did not lag behind that of the controls (Fig. 1). Deaths started to occur on the 3rd day also in this group,

Table III

Lesions in chicken embryos infected with *M. gallisepticum* and in control embryos

	A	P_A	K_A	B	P_B	K_B
Inflammation of the CAM	46/50	3/15	0/15	19/28	2/15	0/15
Heterophil-granulocytic hepatitis	42/50	1/15	0/15	16/28	1/15	0/15
Bronchopneumonia	24/50	1/15	0/15	22/28	0/15	0/15
Heterophil-granulocytic infiltration of blood vessel walls	33/50	0/15	0/15	17/28	0/15	0/15

but while in group A 44% of the total mortality was made up by unhatched embryos, here this value was 90%. By the χ^2 test, the mortality rate found in group B was significantly different from that established for the control group P_B ($P < 0.01$) and K_B ($P < 0.01$). Deaths are given, in a cumulative manner, in Fig. 2.

The gross and histopathological examination of the killed and succumbed embryos revealed changes similar to, but milder than, those found in group A (Table III). The inflammation of the CAM was accompanied by epithelial degeneration and necrosis extending to somewhat smaller areas or cell groups than in group A. In the periportal tissues of the liver, around the major blood vessels there was an inflammatory infiltration consisting of 2 to 5 cell rows. No marked accumulation of bilirubin was observed among the rows of hepatocytes. Bronchopneumonia was restricted to circumscribed areas. By the end of incubation (by PI day 6) also in this group mononuclear cells had appeared among the heterophilic granulocytes in the inflammatory infiltrations occurring in the various organs and around the wall of blood vessels. The incidence of the above lesions was substantially higher than in the control P_B group, but lower than in group A.

In group B 22 chicks hatched. Their growth rate and development were not inferior to those of the controls. Among the hatched chicks no deaths occurred. No pathological lesions were observed in birds killed at various intervals. Histologically, there were focal infiltrations consisting of mononuclear cells (lymphoblasts, lymphocytes and, here and there, histiocytes and plasma cells) in the periportal tissues of the liver, in the interstices of the kidney (Fig. 6) and the lungs and in the wall of some parabronchi (Fig. 7) as well as in the visceral pleura. The follicles of the bursa of Fabricius showed hyperplasia from the 5th day, whereas the Malpighian bodies of the spleen from the 20th day after hatching. In the control groups (group K_A and K_B) one chick died; however, it did not show the above lesions. Some of the embryos inoculated with medium P_A and P_B developed a circumscribed inflammation of the CAM along the channel of inoculation. Three and one of the embryos died in group P_A and P_B, respectively.

In *group C* two chicks died, one on PI day 5 while the other on PI day 6, whereas no deaths occurred at all in group B. Body mass gain did not differ significantly from that of the control ($P > 0.05$). In all the killed chicks of group D, and in some group C chicks necropsied on PI days 20 and 30, the nasal mucosa was reddened, swollen and covered with catarrhal exudate; the other organs were devoid of gross lesions. The histological examination (Table IV) of the above birds revealed that, compared to the controls (Fig. 8), the mucous membrane of the nasal conchae was thickened and contained an infiltration by mononuclear cells (lymphocytes, lymphoblasts, histiocytes and plasma cells), the severity of which varied in the different mucosal segments (Fig. 9). Lymphoid

Table IV

Lesions in day-old chicks infected with *M. gallisepticum* and in control chicks

	Hatched in infected state (B)	C	D	P_C	K_{CD}
Focal lympho-histiocytic pleuritis, airsacculitis and pneumonia	19/22	47/50	26/30	2/20	0/20
Focal lympho-histiocytic infiltrations in the liver and kidney	18/22	44/50	19/30	5/20	6/20
Lympho-histiocytic rhinitis	16/22	6/50	27/30	1/20	1/20

follicles and haemorrhages were frequently observed. In these portions of the mucosa the epithelial cells were degenerated and, in some areas even became detached. The nature and degree of lesions found in the other organs were consistent with those seen in chicks hatched from group-B embryos. In *group D* the incidence of all lesions, whereas in group C that of all lesions except lympho-histiocytic rhinitis was significantly higher than in the control groups P_C and K_{CD} (Table IV). The only difference between birds of group C and group D was that while in group D the lesions of nasal conchae were more expressed ($P < < 0.01$), in group C mononuclear cell infiltrations of the interstices of various organs (kidneys, liver, etc.) occurred more frequently. In group D the above-listed lesions occurred with a similar frequency as in the group of chicks that had hatched out in infected state, while in group C rhinitis was somewhat less frequent ($P < 0.01$). In groups P_C and K_{CD} no deaths occurred. In birds killed in group P_C the above lesions occurred sporadically, whereas in birds of group K not at all.

Electron microscopy revealed that mycoplasmas were present in the samples taken from group A embryos between days 12 and 19 of incubation, in necrotized areas of the CAM, in the lumen of some parabronchi, mainly extracellularly (Fig. 10) and, rarely, in necrotized remnants of epithelial cells, singly or in small groups. No mycoplasmas were demonstrable in organ samples taken from groups B, C and D.

Serological tests

Table V contains the reciprocals of HI antibody titres obtained for groups B, C, D and K_{CD}. Although in the inoculated birds the antibody titres rose parallel with age, their levels remained rather low. Sera of K_{CD} and P_C birds failed to give any positive reaction.

The results of cellular tests are given in Table VI. In the infected group (group C) both tests indicated a more frequent blastogenic response and rosette formation on post-hatching days 10 and 20 than on the 1st day after hatching.

Table V

Results of the haemagglutination-inhibition (HI) test*

Group	Age (days)				
	1	5	10	20	30
"B" hatched chicks	1.41	2.51	3.16	6.32	8.00
"C"		2.83	3.16	4.00	3.16
"D"		2.83	0.00	5.03	8.00
K_{CD}		0.00	0.00	0.00	0.00

* Means of reciprocals of the highest serum dilution giving positive reaction

Table VI

Results of the cellular tests

Time of examination	Immunorosette formation			Lymphocyte-stimulation test		
	Infected group (C)	Control group (P_C)	*t* test	Infected group (C)	Control group (P_C)	*t* test
At hatching	1.4*	—		5.8**	—	
On day 10	9.8	1.66*	NS	19.0	1**	$P < 0.01$
On day 30	14.4	5.0	NS	25.6	7.33	NS

Remarks: NS = not significant; * rosette formation in %; ** blastogenic transformation of lymphocytes in %

Discussion

The present experiments have confirmed that *M. gallisepticum* entering the egg during incubation kills only a part of the embryos. The survivors may hatch in infected state and, as such, play a role in spreading infection.

In group A (embryos inoculated on day 7 of incubation) most of the deaths occurred between the 10th and 13th days of incubation, whereas in group B (embryos inoculated on day 14 of incubation) most of the losses were due to embryos surviving up to the end of incubation but failing to hatch. The non-viability of embryos was due to the lesions which included a diffuse serous-necrotic inflammation of the CAM, bronchopneumonia, heterophil-granulocytic interstitial hepatitis, and heterophil-granulocytic infiltration around part of the blood vessels of the various organs and tissues. These findings are consistent with the results of Goto et al. (1984).

No appreciable mortality occurred among the chicks hatched from group B embryos in infected state and among those inoculated when day-old into the airsac (group C) or intranasally (group D). The pathological lesions occurring in these chicks, as opposed to the diffuse, heterophil-granulocytic and expressed exudative histological alterations of the embryos, showed focal and productive

character; the infiltrating cells consisted mostly, or exclusively, of lymphocytes, lymphoblasts, histiocytes, and a few plasma cells. Similarly to the findings of others (Kirklyn and Olson, 1967; Grimes and Rosenfeld, 1972), these lesions were observed in the walls of the airsacs, in the lungs and in the interstitium of the liver and kidneys.

All these facts suggest that in the embryos the lesions, which are indicative of an impaired protective mechanism, allow the pathogenic agent to proliferate and become widespread in the whole organism. On the other had, in the chicks the nature and cell composition of the changes reflect the appearance of an enhanced cell-mediated immunity; even plasma cells, indicative of antibody production, could be observed. In spite of this, the widespread histopathological (liver, lungs, kidneys) alterations found in birds of group B and C, as well as the isolation of mycoplasmas from the heart blood of one bird of group B and C each on PI day 5, suggest that mycoplasmas spread via the blood stream also in chicks (mycoplasmaemia).

We found that the cell composition of responses to experimental viral (lentogenic Newcastle disease virus and avian reovirus) infections (Glávits et al., 1983, 1984*a*) and bacterial (*Salmonella typhimurium* and *Staphylococcus aureus*) infections (Glávits et al., 1984*b*) correlated with the developmental status of the chicken embryos' myeloid and lymphoid apparatus (Glávits et al., 1981). The embryos' and chicks' response to *M. gallisepticum* infection resembled in cell composition and character the response to the above viruses; however, there was difference in cytopathic effect and distribution of lesions in the organs.

By electron microscopy, Boam and Sanger (1970) observed mycoplasmas both extracellularly, among the microvilli of the epithelial cells of the infected mucosa, and intracellularly. We found the pathogens on the surface of the CAM and in the lungs of the embryo, mostly in extracellular location; intracellularly the agent was found only in the remnants of detached and necrotized epithelial cells.

As opposed to the control, lymphocytes separated from the peripheral blood of the infected chicks on PI days 10 and 30 recognized the antigen and responded to antigenic stimulus with blastogenic transformation and rosette formation. The level of HI antibodies rose parallel with age but remained at a low level throughout. Therefore, the diagnostic value of the HI test in the early recognition of mycoplasma infection of embryos and day-old chicks is, in our view, doubtful.

Our results, in accordance with those of other authors (Jordan, 1981; Luginbuhl et al., 1967), suggest that cell-mediated immunity has an essential role in controlling mycoplasma infection. Further, the results of Parry and Aitken (1973) underline the importance of local immunity and IgA production in the protection against respiratory infection caused by mycoplasmas present on the surface of mucous membranes.

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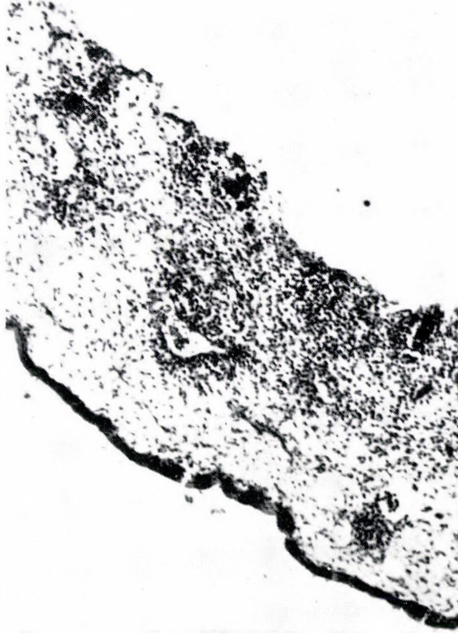


Fig. 3. Perivascular inflammatory infiltration in the oedamatously-thickened CAM (group A) and under the necrotic and detached epithelial layer. Haematoxylin and eosin (H.–E.), × 90

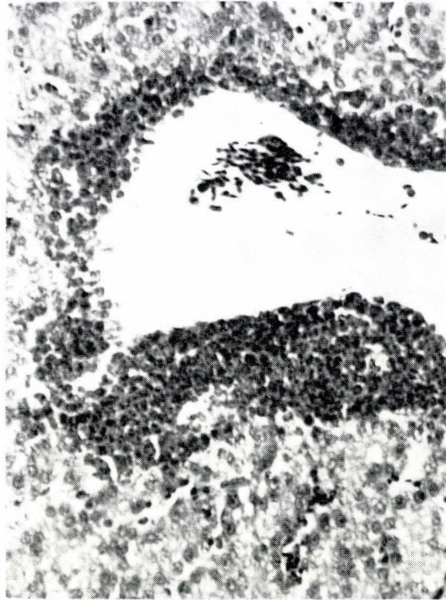


Fig. 4. Heterophil-granulocytic infiltration in the periportal areas of the liver from a succumbed embryo (group A). H.–E., × 160

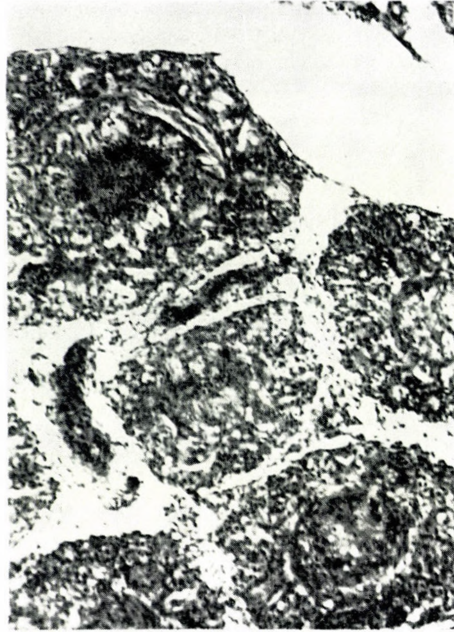
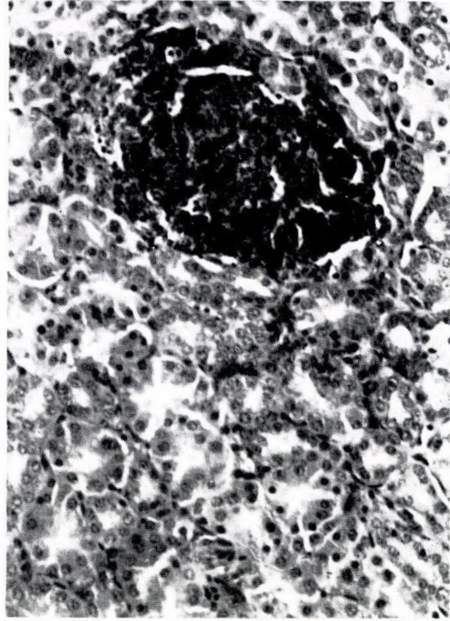
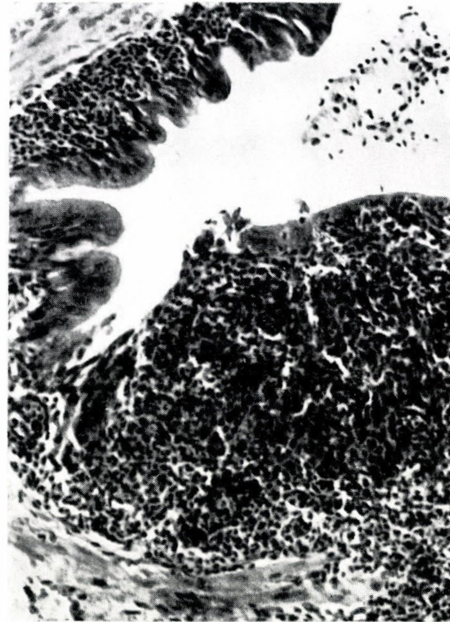


Fig. 5. Inflammation accompanied by granuloma formation in the lumen of, and around, parabronchi in the lungs from a group-A embryo that died on PI day 14. H.–E., × 90





Figs 6 and 7. Focal infiltrations by mononuclear cells in the kidneys (Fig. 6) and lungs (Fig. 7) from a chick hatched in infected state. H.–E., × 160

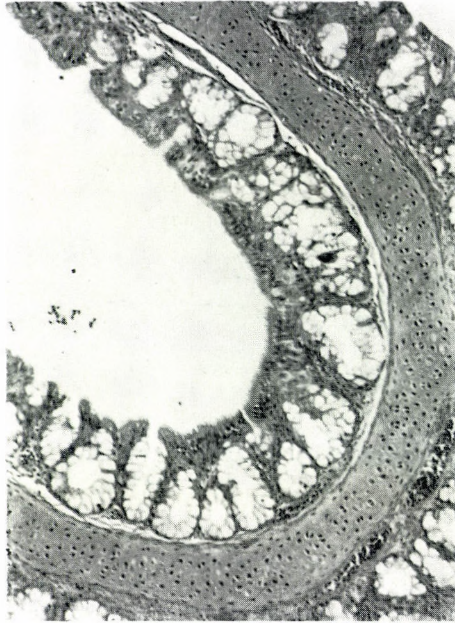


Fig. 8. Nasal turbinate portion from a control chick. H.–E., × 90

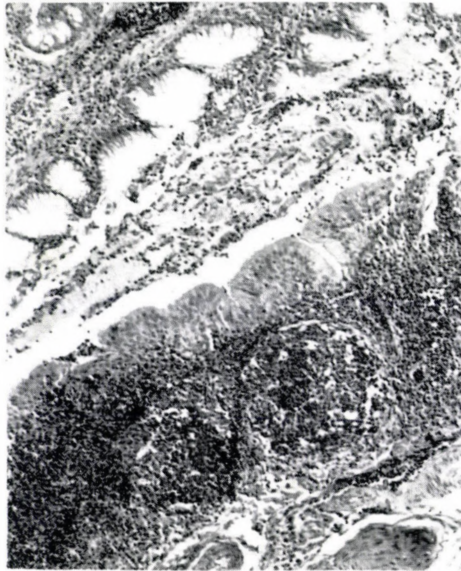


Fig. 9. Nasal turbinate portion from an intranasally-inoculated chick (group D) on PI day 20. The mucous membrane is infiltrated by mononuclear cells and covered with exudate. H.–E., × 90

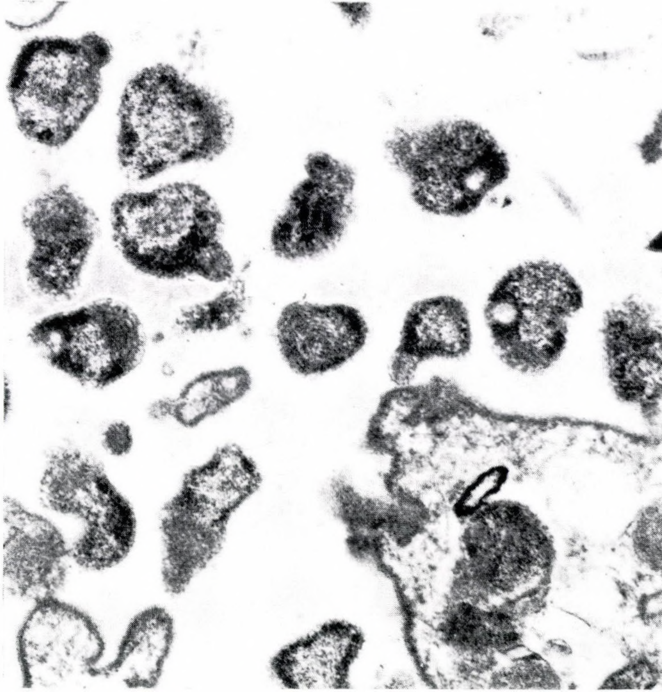


Fig. 10. Mycoplasmas in the lumen of parabronchi from a chicken embryo (group A) that died on PI day 12. Electron micrograph, × 45,000

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GROSS AND HISTOPATHOLOGICAL STUDY OF EXPERIMENTAL *MYCOPLASMA* MASTITIS OF CATTLE

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A total of 11 udder quarters of 4 cows were inoculated intracisternally with *Mycoplasma bovis* cultures. Four to 60 h post-infection (PI), part of the affected udder quarters were sampled by needle biopsy for histological and electron-microscopic examination. The cows were slaughtered on PI days 6, 8, 14 or 28. Within 17 to 60 h gross and histopathological changes characteristic of a more or less severe, acute, purulent, parenchymal mastitis developed in the infected udder quarters.

Six days PI the infected udder quarters were swollen, their cut surface was yellowish-brown and granular. The lumen of glandular alveoli was filled up with lipid droplets and neutrophilic granulocytes.

Eight days after infection condensed yellowish-white pus droplets could be squeezed out onto the cut surface of udder quarters and the milk cistern was also filled with condensed, purulent and fibrinous exudate. The lymph nodes were swollen, while the interalveolar connective tissue was infiltrated by lymphocytes, plasma cells and neutrophilic granulocytes.

By the 14th day PI the interalveolar connective tissue had undergone proliferation and showed a diffuse infiltration by plasma cells, lymphoid cells and granulocytes. The glandular alveoli had ceased to secrete milk. The glandular substance of the udder had become tough and connective tissue outgrowths had appeared on the milk cistern mucosa.

Four weeks PI both the infected and the uninfected udder quarters were tough, firm, and the mucous membrane of the milk cistern was thickened and uneven. Histologically, an expressed proliferation of the interalveolar connective tissue, diffuse infiltration of the connective tissue by plasma cells, lymphoid cells and granulocytes, presence of a few neutrophilic granulocytes in the atrophied glandular alveoli, and transformation of the milk cistern mucosal epithelium into stratified squamous epithelium were observed. In the supramammary lymph nodes a more or less severe hyperplasia and inflammation occurred already from PI day 6. Attempts to demonstrate mycoplasmas in the udder and lymph nodes by electron microscopy at various times (24 h, 8 days, 14 days) PI consistently failed.

Keywords. *Mycoplasma bovis*, experimental, mastitis, cow, bovine, pathology, histopathology.

Bovine mastitis caused by *Mycoplasma bovis* was first reported in 1962 by Hale et al., who also isolated the pathogen from the udder of affected cows. By inoculating the secretion of pathologically-altered udder quarters, or the *M. bovis* cultured from the former on blood agar, into the milk cistern of cows, they succeeded in producing mastitis. At necropsy, nodules 2 mm in diameter were found on the mucous membrane of the milk cistern, and fibrinous or condensed purulent contents in the milk ductules. Histologically, purulent mastitis was

accompanied by hyperplasia of the epithelium of milk ductules, proliferation of connective tissue elements and granuloma formation.

Kehoe et al. (1967) infected 8 cows and monitored, by histological examination, the time-course of the development of lesions. The appearance of neutrophils and of an exudate showing eosinophilia in the alveolar lumina of the lactiferous gland was observed already 1 or 2 days after infection. Four to six days PI, in addition to the above changes, an interstitial infiltration by neutrophilic and eosinophilic granulocytes, reticulocytes, lymphocytes and plasma cells was also prominent. After 2 weeks, here and there the alveolar epithelium showed necrosis and detachment of cells, while in other places slight metaplasia. Three weeks after infection, besides alveolar atrophy, a mild fibrosis of the udder parenchyma was also present. In the cortex of supramammary lymph nodes the formation of numerous secondary lymph follicles and an infiltration by neutrophilic and eosinophilic granulocytes as well as plasma cells were observed.

Karbe et al. (1967) inoculated *M. bovis* cultures intracisternally into 17 udder quarters of 7 cows, and monitored the time-course of development of gross lesions and histopathological changes in animals slaughtered at different intervals between PI days 1 and 203. In cows slaughtered between PI days 1 and 3 the udder was swollen, its cut surface was ochre-coloured and its substance easy to incise. The supramammary lymph nodes were moderately swollen and had a juicy cut surface. Within 4 to 10 days of infection the udder became compact to the touch and the yellow glandular lobules stood out from its cut surface. Within 2 to 3 weeks nodules approximately 2 mm in diameter appeared on the milk cistern mucosa, the secretion formed plugs in the milk ductules, and a yellowish, turbid exudate could be squeezed out on the cut surface of the udder. After a period of 4 to 6 weeks the udder became markedly compact, in places firm, to the touch. The supramammary lymph nodes were greatly enlarged and their substance protruded over the cut surface.

In the early stage, histological examination demonstrated in the parenchyma an infiltration by eosinophilic granulocytes instead of neutrophilic ones. The advanced stages of mastitis were characterized by proliferation, galactophoritis, and atrophy of the glandular substance.

By intracisternal inoculation of the toxin obtained from sonicated cultures of *M. bovis*, Karbe and Mosher (1968) produced mastitis accompanied by eosinophilic infiltration. By the sixth h after infusion of the toxin the udder quarter had become swollen and compact to the touch. Milk leucocyte count had risen strikingly (to 5×10^6/ml) and returned to the initial value only 5 days later. By immunofluorescence, Karbe and Mosher (1968) demonstrated mycoplasmas in the cytoplasm of granulocytes, macrophages and alveolar epithelial cells in cows infected intracisternally with *M. bovis* 24 h earlier. No mycoplasmas were present in the interstitium.

Buchvarova and Veselinova (1973) experimentally infected 18 cows with several *Mycoplasma* strains including *M. bovis*, and succeeded in producing clinically apparent or subclinical mastitis. Histologically they found an infiltration of the glandular substance and the interstitium by eosinophilic granulocytes and lymphoid cells.

In a review article, Boughton (1979) summarized the publications that had appeared on *M. bovis* mastitis up to 1978. According to him, the microscopic pathological changes in spontaneous infections have been described by only a few workers (Hale et al., 1962; Rinaldi et al., 1969; Redaelli and Ruffo, 1975; Bennett and Jasper, 1978).

Seffner and Pfützner (1980) report gross and histopathological findings obtained on a total of 60 cows slaughtered 2 to 9, 14 or 21 days after experimental infection. Within 2 to 9 days the udder became enlarged and compact to the touch, its cut surface was grayish-yellow and granular. The histological picture was dominated by severe degeneration and infiltration of the alveolar epithelium by neutrophils. By the 14th and 21st day PI the udder quarters had become smaller, more compact and brownish-yellow. The alveoli were atrophied, less abundant in neutrophilic granulocytes than earlier, and in the interalveolar connective tissue there was an infiltration by plasma cells.

By ultrastructural study of a total of 16 udder quarters experimentally infected with *M. bovis* culture, Stanarius et al. (1981) found mycoplasmas in 4 quarters, either phagocytosed by neutrophilic granulocytes present in the alveoli or surrounded by pseudopodia on the surface of granulocytes.

Materials and methods

The isolation of *M. bovis* and the intracisternal inoculation were described in detail by Horváth et al. (1980, 1981, 1983). In addition to the experiments presented there, a further experimental infection has been performed. All udder quarters and supramammary lymph nodes of four cows were subjected to gross and histopathological examination. Histological samples were taken partly from the udders of living animals by needle biopsy, partly during the necropsy of slaughtered cows and the examination of their pathologically-altered udder quarters. Tissue pieces excised from altered areas of the udder were fixed in 10% formalin, embedded in paraffin, and the sections were stained with haemalaun and eosin. Occasionally, to demonstrate lipid materials in frozen sections, the Oil-Red-O stain was used, too.

The experimental animals were grouped according to the time elapsed from infection to slaughter, as follows:

Experiment 1. A six years old Holstein-Friesian cow (ear tag no.: 529), being in the 5th month of lactation was given, into both of her right udder quar-

ters, 5 ml of the broth culture of a mixture of *M. bovis* strains (containing equal amounts of strains no. 1199, 1200, 1201 and 5043). The mixture contained 2×10^6 colony-forming units (CFU)/ml. The cow was slaughtered 6 days PI.

Experiment 2. A 4 years old Hungarian Fleckvieh cow (ear tag no.: 590152), which was at the end of lactation, received into each of her four udder quarters 5 ml of *M. bovis* broth culture (strain 5063), containing 10^4 CFU/ml. Biopsy samples were taken 17, 23, 48 and 60 h PI. The cow was slaughtered 8 days PI. After slaughter, samples were taken from the udder and supramammary lymph node of this cow for electron microscopy.

Experiment 3. A Hungarian Fleckvieh cow (ear tag no.: 520333), being at the end of lactation, was injected, into each of her four udder quarters, with 5 ml of a *M. bovis* broth culture containing 10^4 CFU/ml. Biopsy samples for histological examination were taken 4, 24, 30 and 52 h PI, and the cow was slaughtered on PI day 14. The biopsy material obtained 24 h PI and the samples taken from the mammary gland and supramammary lymph nodes after slaughter were examined also by electron microscopy.

Experiment 4. A Hungarian Fleckvieh cow (ear tag no.: 387), being in the 7th month of lactation, was given, into the left rear udder quarter, 5 ml of the 10^{-2} dilution of a *M. bovis* (strain 5059) culture containing 3×10^7 CFU/ml. Samples for light microscopy were taken 4 weeks PI, at slaughter.

Results

Experiment 1

The right udder quarters of the cow slaughtered 6 days PI were swollen, light yellowish-brown, compact to the touch and easy to incise. On section, the cut surface appeared granular, and pus droplets and a reddish secretion resembling curdled milk could be squeezed out onto it (Fig. 1). Onto the light rose-coloured cut surface of the left udder half large quantities of milk could be squeezed out. The right supramammary lymph node was markedly swollen and had a juicy cut surface.

Histologically, in the infected quarters the milk cistern mucosa was infiltrated by lymphocytes and neutrophilic granulocytes. Most of the milk ductules and glandular alveoli were filled with serous exudate showing eosinophilia, neutrophilic granulocytes and detached epithelial cells (Fig. 2). Here and there the alveolar epithelium was badly swollen and vacuolated. The luminal border of the epithelial cells was indistinct and most of the lumen was filled with lipid droplets staining with Oil-Red-O. The interstitium was broadened and slightly to moderately infiltrated by lymphocytes and neutrophilic granulocytes (Fig. 3). In the parenchyma of the left udder half, besides intact and functioning alveoli, there were groups of alveoli which had dried off and con-

tained a few neutrophilic granulocytes. The cortical nodules of the right supramammary lymph node had become enlarged. In the germinal centre there were large blast cells poor in chromatin and having a spherical nucleus; several dividing stages were noted among them. Around the germinal centre there was a dense lymphocytic ring (Fig. 4). The cells of the medullary cords underwent proliferation and varying numbers of plasma cells appeared among them (Fig. 5). The cells lining the medullary sinuses were swollen and most of them had detached from the sinus wall.

Experiment 2

The histological examination of biopsy samples taken from all the four udder quarters of cow no. 590152 in PI hours 17, 23, 48 and 60 revealed that 17 h after infection the alveoli contained small amounts of serous exudate, a few detached alveolar epithelial cells and one or two neutrophilic granulocytes. In samples taken in the 23rd h PI some alveoli exhibited slight epithelial damage (detachment of the epithelium) and oedema of the interlobular septa. By PI h 48 an expressed infiltration by neutrophilic granulocytes, vacuolation of epithelial cells and, in the interstitium, oedema and focal infiltration by neutrophilic granulocytes had developed. In the biopsy sample taken from the udder in the 60th h PI the alveolar epithelium exhibited severe vacuolation and in the alveolar lumina there were intact as well as disrupted neutrophilic granulocytes and small amounts of serous exudate showing eosinophilia.

The cow was slaughtered 8 days after infection. All the four udder quarters were moderately swollen, compact to the touch and had a light yellowish-brown cut surface. On the cut surface numerous yellowish-white, dense pus droplets and cylinders of cheese-like consistence could be squeezed out. The milk cistern was filled with a thick, yellowish-white exudate, the mucosa of the milk cistern was slightly hyperaemic and swollen but smooth. The supramammary lymph node was swollen and its cut surface was juicy (Fig. 6).

Histologically, the glandular alveoli were mostly filled with a serous exudate showing eosinophilia, and contained neutrophilic granulocytes and lipid materials; a few alveoli contained corpora amylacea as well. In the interstitium there was an expressed infiltration by lymphocytes, granulocytes and plasma cells (Fig. 7). The mucosae of the larger milk ducts and milk cistern were infiltrated with neutrophilic granulocytes and plasma cells; in the mucosal epithelium signs indicative of proliferation were seen. The microscopic pathological changes of the supramammary lymph node were identical with those found in Experiment 1.

Eight days after infection no mycoplasmas were seen by electron microscopy in the udder and supramammary lymph node.

Experiment 3

Biopsy samples were taken from all the four quarters of cow no. 520333 4, 24, 30 and 52 h PI. No histopathological alterations were seen in the udder tissue obtained by biopsy 4 h after infection. After 24 and 30 h, in part of the alveoli there was a mild to moderate infiltration by neutrophilic granulocytes, slight to expressed vacuolation of the alveolar epithelium, and interstitial oedema. Fifty-two h after infection in the alveolar epithelium, here and there, vacuolation, in the lumen of alveoli a more or less severe infiltration by neutrophilic granulocytes and eosinophilic serous contents, whereas in the intralobular connective tissue oedema and moderate infiltration by neutrophilic granulocytes were seen. Both udder halves of the cow, slaughtered 2 weeks after infection, were moderately enlarged, compact to the touch, their cut surface was light brownish and in some places pus droplets could be squeezed out on it. The milk cistern contained a yellowish-white, debris-containing exudate. On the milk cistern mucosa compact, grayish-white nodules the size of a millet or pea were observed. The udder substance was tough, firm, and hard to tear. The supramammary lymph nodes were slightly enlarged, compact to the touch, their cut surface was light brown, succulent, and contained no foci visible with the unaided eye.

Histologically, the alveolar epithelium did not show secretory activity, the alveolar lumina had narrowed down and contained a few neutrophilic granulocytes and, here and there, small amounts of serous exudate. The interalveolar tissue markedly increased in quantity and, together with the mucous membrane of the milk cistern, it was infiltrated by granulocytes and plasma cells (Fig. 8). On the mucous membrane proliferative outgrowths were seen. The mucosal epithelium was a stratified squamous epithelium (Figs 9 and 10). In the cortex of lymph nodes the cortical nodules were moderately hyperplastic. Most of the cells of the hyperplastic medullary cords were plasma cells. In the serous exudate present in the dilated sinuses, besides numerous neutrophilic granulocytes, there were detached sinus cells and a few plasma cells.

By electron microscopy we failed to demonstrate mycoplasmas in the biopsy material taken from the udder 24 h PI and in the udder and supramammary lymph node examined 14 days PI.

Experiment 4

The left rear (infected) udder quarter as well as the other three quarters (control) of the cow slaughtered 4 weeks after infection were moderately swollen, compact and firm to the touch, their cut surface was light grayish-brown, smooth and exhibited irregular grayish-white motives. Small amounts of turbid serous or purulent exudate could be squeezed out on the cut surface. The sub-

Fig. 1. The cut surface of the udder is finely granular, and pus droplets can be squeezed out on it (PI day 6)

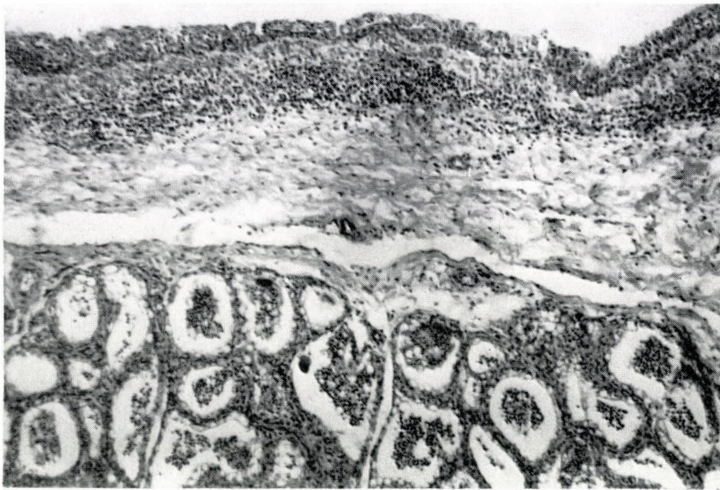

Fig. 2. The mucous membrane of the milk cistern is infiltrated by inflammatory cells; in the alveoli groups of neutrophilic granulocytes are seen (PI day 6). H.–E., × appr. 80.

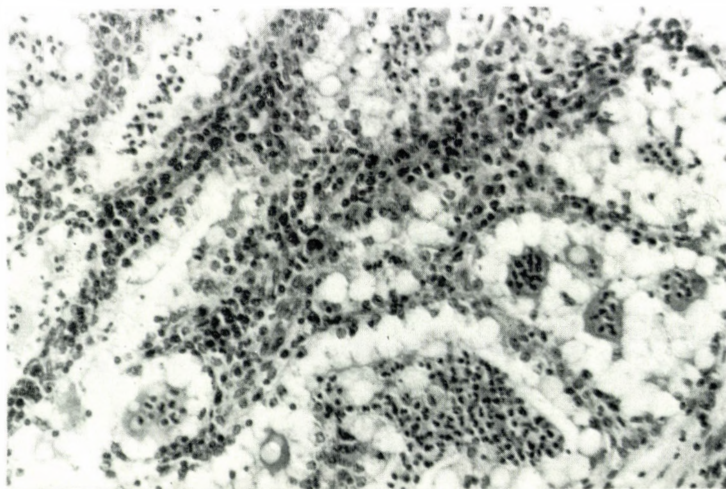

Fig. 3. The lumen of the alveoli is filled with lipid droplets and granulocytes; in the interstitium there is an infiltration by lymphocytes and neutrophilic granulocytes (PI day 6). H.–E., × appr. 200

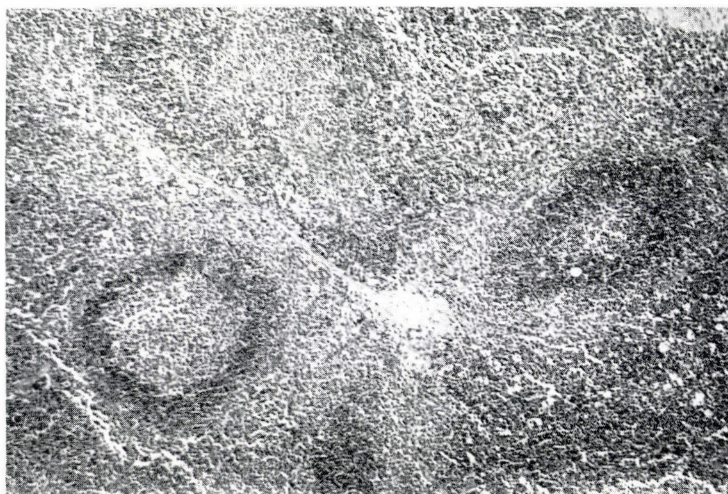

Fig. 4. The cortical nodules of the supramammary lymph node are hyperplastic (PI day 6). H.–E., × appr. 63

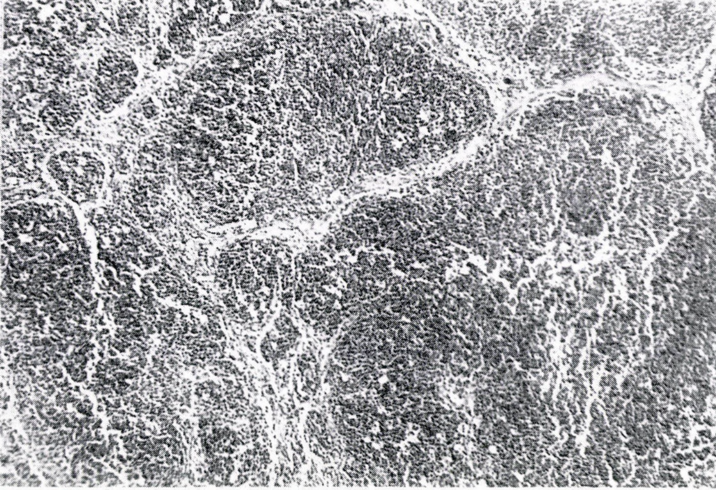

Fig. 5. The cells of the medullary cords of the lymph node have increased in number. The medullary cords are infiltrated by varying numbers of plasma cells (PI day 6). H.–E., ×appr. 63

Fig. 6. The milk cistern is filled with yellowish-white purulent or fibrinous exudate. The supramammary lymph node is swollen (PI day 8)

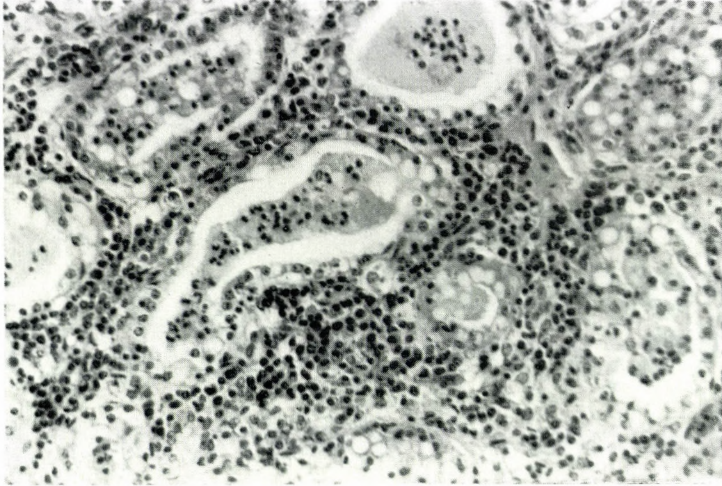

Fig. 7. The interalveolar interstitium is infiltrated by lymphocytes, plasma cells and granulocytes (PI day 8). H.–E., × appr. 200

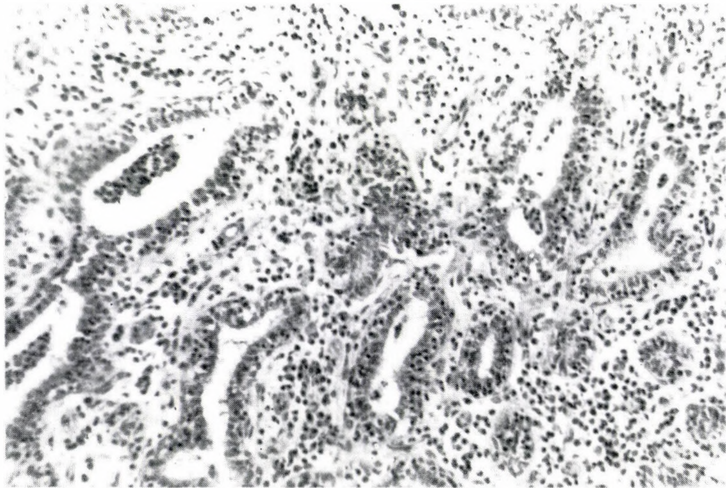

Fig. 8. Two weeks after infection the interalveolar connective tissue of the mammary gland is markedly increased and infiltrated by inflammatory cells. The alveoli do not show secretory activity. H.–E., × appr. 160

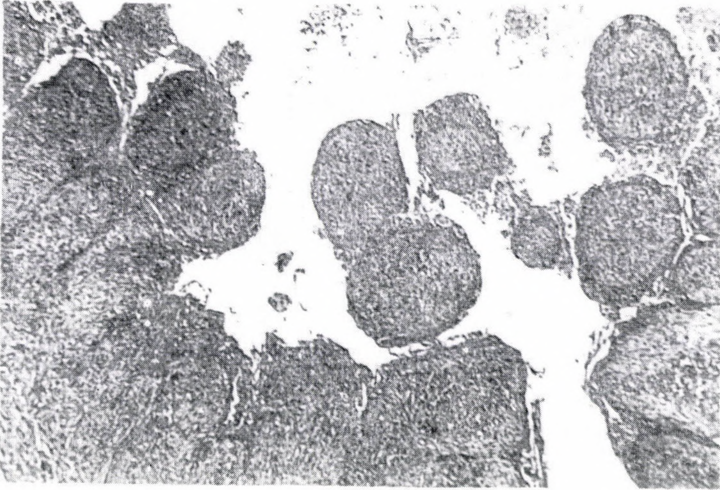

Fig. 9. Tuberous connective-tissue outgrowths on the mucosa of the milk cistern (two weeks PI). H.–E., × appr. 50

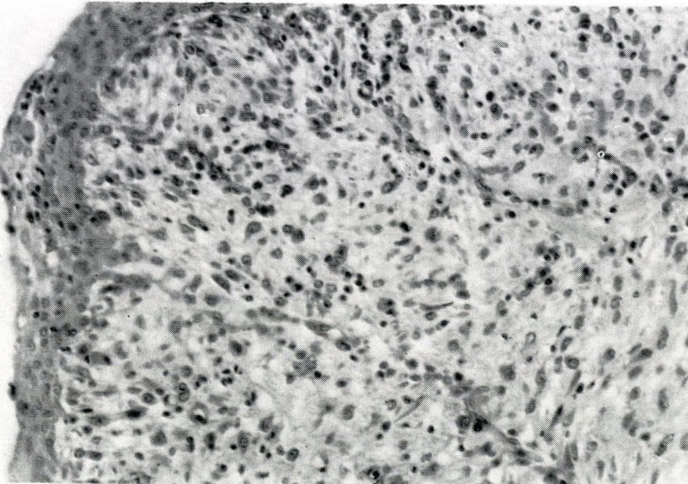

Fig. 10. The epithelium of the outgrowths is metaplastic (two weeks PI). H.–E., × appr. 200

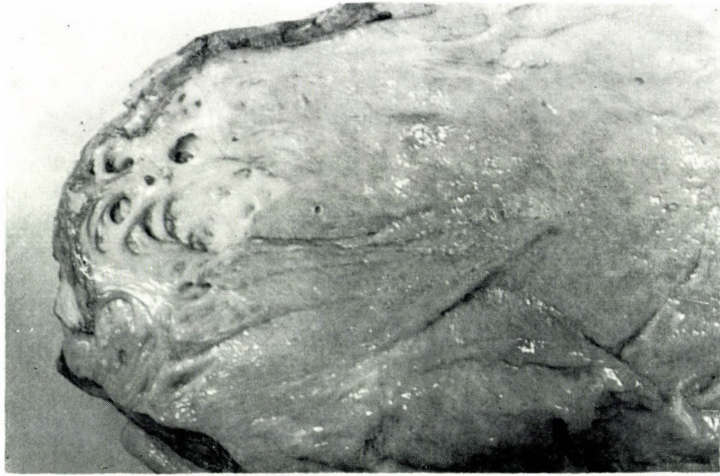

Fig. 11. Four weeks after infection the substance of the udder is firm; on the mucous membrane of the milk cistern connective-tissue growths can be seen

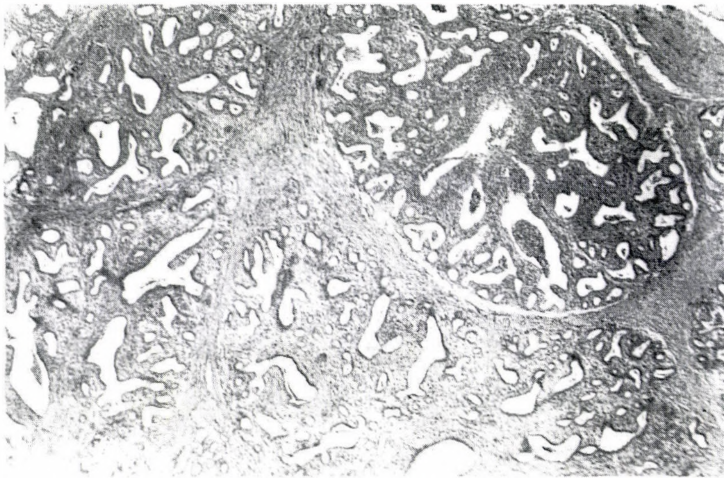

Fig. 12. The alveoli do not show secretory function, the intraalveolar and interalveolar connective tissues have undergone marked proliferation (4 weeks PI). H.–E., × appr. 25

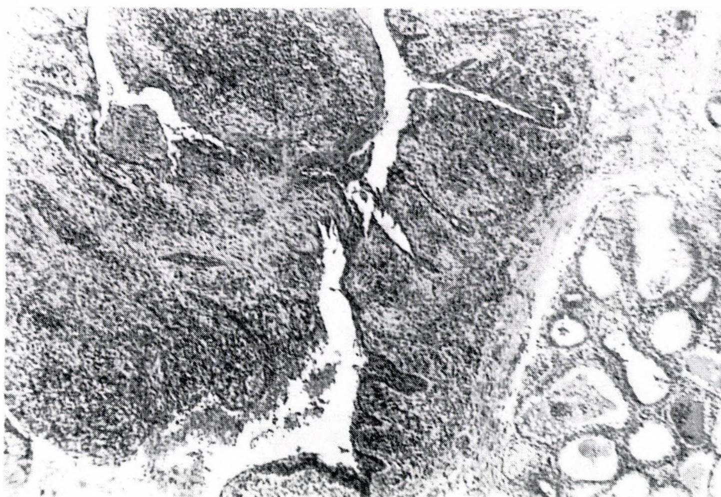

Fig. 13. The mucous membrane of the milk cistern and the connective-tissue growths are infiltrated by plasma cells and lymphocytes. The epithelium is a metaplastic, stratified squamous epithelium (4 weeks PI). H.–E., × appr. 50

Fig. 14. Tertiary follicle in the cortex of the supramammary lymph node (4 weeks PI). H.–E., × appr. 80

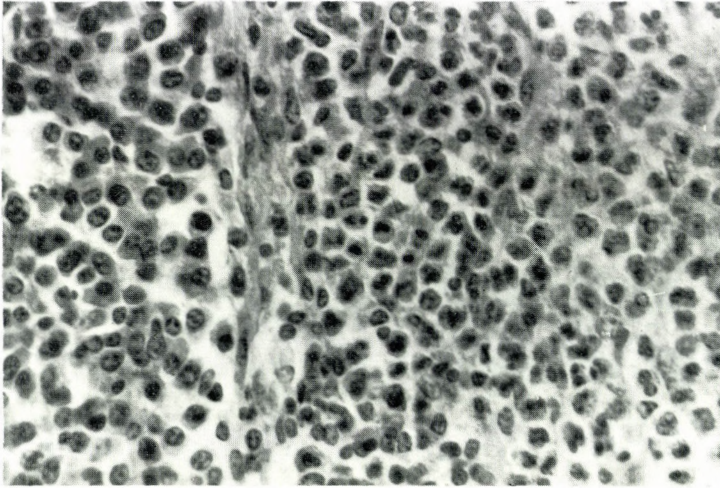

Fig. 15. Diffuse infiltration by neutrophilic granulocytes in the medullary sinuses of the supramammary lymph node (4 weeks PI). H.–E., × appr. 500

stance of the udder was tough, firm, hard to tear (Fig. 11). The mucous membrane of the milk cistern was thickened, uneven and tubercled, and the supramammary lymph nodes were markedly swollen.

Histological examination revealed that the glandular alveoli did not secrete, were atrophied, and contained little serous exudate and, some of them, neutrophilic granulocytes. The interlobular collagenic connective tissue and septa were markedly widened, while in the interlobular and intralobular connective tissue proliferation of fibroblasts and fibrocytes and diffuse infiltration by plasma cells, lymphoid cells and neutrophilic granulocytes were seen (Fig. 12). The epithelium of the milk cistern mucosa and of the mucosa lining the connective tissue outgrowths seen in the milk ducts was a stratified squamous epithelium infiltrated by neutrophilic granulocytes. The lamina propria of the milk cistern mucosa and the connective tissue framework of the outgrowths were infiltrated primarily by plasma cells and lymphoid cells (Fig. 13). In the cortex of the supramammary lymph node large tertiary follicles were seen (Fig. 14), together with signs indicative of mitosis also outside the follicles. In the areas between the medullary cords there was a severe diffuse infiltration by neutrophils (Fig. 15).

Discussion

On the basis of the four experiments we made an attempt to reconstruct how the lesions developed in the experimentally infected cows. Four h PI no histopathological changes are yet present in the udder tissue. Seventeen h PI the alveoli contain small amounts of serous exudate and a few neutrophils as well as detached epithelial cells. By PI h 23–60 a moderate to severe infiltration by neutrophils develops in the alveoli. The early changes observed by us are consistent with those reported by Kehoe et al. (1967) and Seffner and Pfützner (1980), but differ from those described by Karbe et al. (1967), Karbe and Mosher (1968) and Buchvarova and Veselinova (1973): namely, these latter authors found an infiltration by eosinophilic granulocytes instead of neutrophils. According to Seffner and Pfützner (1980), in cases of mastitis predominantly degranulated neutrophils are found, since during their phagocytic activity the cells use lysozyme and, as a result, they become less granulated. In acute *Mycoplasma* mastitis well-granulated, segmented cells are present together with degranulated ones. When segmented cells occur at a high density, the granules are hard to observe. During the first five days predominantly neutrophilic granulocytes were found also in the milk sediment (Jain et al., 1969; Seffner and Pfützner, 1980). The granules of neutrophils are finer and stain lighter than those of eosinophils, which appear orange red in Giemsa preparations. During the electron-microscopic study of acute *Mycoplasma*

mastitis, Stanarius et al. (1981) never found in the alveolar lumina granulocytes whose granules resembled those of eosinophils.

Gross lesions and histopathological changes characteristic of acute purulent galactophoromastitis develop in 6 to 8 days' time. At that time signs indicative of hyperplasia are seen in the supramammary lymph nodes.

Two weeks after infection a subacute or chronic proliferative inflammation of the mucosa of the milk cistern and milk ducts, atrophy of the alveolar epithelium, proliferation and inflammatory infiltration of the interalveolar connective tissue can be observed. The supramammary lymph nodes exhibit subacute inflammation, with a marked increase of the number of plasma cells in the medullary cords.

Four weeks PI the granulomatous inflammation of the milk duct mucosae is even more severe. In addition to alveolar atrophy, a chronic proliferative inflammation and fibrosis of the interstitium, and chronic purulent inflammation of the supramammary lymph node are seen. We have not found eosinophilic granulocytes in any of the inflammatory lesions. No mycoplasmas could be detected by electon microscopy in the udder of a cow examined on PI days 1, 2 and 8.

The subacute or chronic udder lesions that developed in 2 to 4 weeks' time are consistent with those reported by others (Kehoe et al., 1967; Seffner and Pfützner, 1980).

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MEDICATION OF INFLAMMATION OF THE PHALLUS IN GEESE

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Treatment with antimycoplasma antibiotics reduced the clinical appearance of phallus inflammation from 35.27–71.02% to 19.89–34.91% in goose flocks showing a high (about 70%) incidence of mycoplasma infection. The flocks were treated with Lincospectin (Upjohn S. A., Puurs, Belgium; 0.25–0.43 g/bird), Tylan® solubile pulvis ad us. vet. (G. Richter Pharmaceutical Works, Budapest, Hungary, 0.36–0.43 g/bird), Dynamutilin (Hage, Karcag, Hungary, 0.14–0.21 g/bird) in the drinking water over 2 to 6 day sor with Tiamutin® water-solubile 80% ad us. vet. (Biochemie Gesellschaft mbH, Wien, Austria) mixed in the feed in a concentration of 200–300 mg/kg feed over periods of 10 days in the different trials conducted. The number of ganders affected with clinically apparent inflammation of the phallus underwent a 50% decrease in the treated groups. Also, there was a significant difference both in mycoplasma isolation rate (control group: 64.2–76.38%; treated group 40.0–62.67%), and in seropositivity (control: 27.10–67.29%; treated: 12.04–50.92%), in favour of the treated groups. Treatment resulted in a lower egg infertility rate, higher egg production (per layer), and higher hatching rate (by 2.5–7.8%, 1.05–3.5 eggs per layer, and 1.39–7.90%, respectively).

Keywords. Phallus, inflammation, goose, gander, mycoplasma, medication, antibiotics.

Since the first description of inflammation of the phallus in geese (Palya, 1971), contradictory theories have been put forward as regards both the morphogenesis (Dobos-Kovács et al., 1985; Póka et al., 1985) and the aetiology of the disease. Szép et al. (1973) and Pataky (1973) considered it an infectious, while Rénes and Szalay (1973) a non-infectious disease.

Recently, Stipkovits et al. (1984) have reported isolation of mycoplasmas in association with phallus inflammation. With regard to this fact, in the affected goose flocks it seemed expedient to conduct medication trials with antimycoplasma drugs that had proved effective against mycoplasmoses of other animal species, i.e. with Tylan, Lincospectin, Dynamutilin and Tiamutin. These experiments are reported in the present paper.

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Materials and methods

Goose flocks

The trials were conducted in goose-breeding farms where inflammation of the phallus had occurred regularly and several mycoplasma strains had been isolated from the phallic lymph. The sensitivity of the isolated strains to antimycoplasma drugs was examined in preliminary laboratory tests. Five medication trials were performed. Table I shows the number of layers and ganders in the experimental and control groups, the data of the start and the end of the experiments, the drugs applied as well as their mode of application and dose.

The goose flock kept in the same building was divided into an experimental (treated) and a control group. To avoid mixing of the two groups, the two parts of the building and the runs belonging to them were separated with a wire meshing. The two groups were managed and fed in the same manner.

Prior to the experiment, the ganders were examined individually. The birds showing clinical symptoms indicative of inflammation of the phallus were removed from the flock. To compensate for the removed ganders, healthy replacement ganders were added to the group.

Medication

The birds of the experimental groups were treated with different doses of the antibiotics listed in Table I via drinking water or feed, over various periods. The birds of the control group received no treatment.

Monitoring the efficacy of medication

The efficacy of medication was followed up by examining several parameters.

Clinically apparent disease. The gander flock was examined individually, usually at one-month intervals. The birds exhibiting symptoms indicative of inflammation of the phallus were recorded and removed from the flock. As far as possible, the removed birds were replaced by healthy ganders.

Infertility and hatching rate of eggs. The infertility rate and the hatching rate of eggs collected in the experimental and control groups were checked for each batch of eggs placed in the incubator, and were calculated for the whole production period.

Egg production. The number of eggs produced in the period between the incubation of two consecutive batches of eggs was recorded separately for the two groups in each experiment, and was expressed for one layer. In addition, the number of eggs per layer in the whole production period was determined.

Table I

Design of the experiments

	Groups	Layers	Ganders	Drug, applied in drinking water	Date of treatment and dose (g/bird)
I	Treated	1066	320	Lincospectin	30 March–2 April (4 days), 0.43 g/bird
				Tylan	22–24 April (3 days), 0.36 g 11–13 May (3 days), 0.43 g
	Control	1245	320		
II	Treated	1096	382	Dynamutilin	26–29 June, 27–30 July, 20–23 August, 14–17 September, 11–14 October, 11–14 November, 10–13 December (4 days each); 0.21 g
	Control	1075	418		
III	Treated	1200	350	Dynamutilin	24–31 January (6 days), 0.14 g;
				Lincospectin	28 February–2 March (3 days), 0.43 g; 28 March–2 April (6 days), 0.21 g;
	Control	1510	380		28 April–2 May (5 days), 0.25 g
IV	Treated	1034	414	Lincospectin	5–6 September (2 days), 0.36 g; 8–9 Sept. (2 days), 0.36 g; 28 Oct.–1 Nov. (4 days), 0.36 g; 24–25 October (2 days), 0.32 g
	Control	1566	398		
V	Treated	1828	558	Tiamutin (in the feed)	20 Feb.–1 March (10 days), 300 mg/kg feed; 20–30 March (10 days), 200 mg/kg feed
	Control	1723	533		

Mycoplasma isolation and serological examinations. In experiment I, mycoplasma isolation was attempted from the phallic lymph of 20 experimental and 20 control ganders on days 14 and 50 after beginning of the first treatment. The samples were collected according to Schalkház et al. (1982). The examinations were performed as described previously by us (Stipkovits et al., 1975). Mycoplasma isolates were identified by epifluorescence (Varga and Stipkovits, 1984). In experiment IV, 20 experimental and 20 control ganders were selected from which phallic lymph samples were collected at monthly intervals throughout the production cycle. Mycoplasma isolation from these samples was attempted as described above. Serum samples were taken from the same birds, also at monthly intervals, and were checked by the indirect haemagglutination test. For this purpose, antigens were prepared from strains M. sp. 1220 and M. sp. 1221, two strains having different biochemical and serological properties. The test was done according to the method of Cho et al. (1976).

Statistical evaluation

The results obtained for the tested and control groups were compared by the χ^2 test. As regards the number of affected and succumbed birds, the latter was related to the number of geese at the beginning of the experiment. Egg production was evaluated by relating the number of eggs collected between the start of two consecutive incubations to the number of "egg days" (number of layers × number of days) for the given period. Egg production for the whole production cycle was evaluated likewise. To assess infertility rate and hatching rate, the number of infertile eggs and that of hatched eggs were compared to the number of eggs placed in the incubator.

Results

Experiment I

From the start of the experiment up to the first clinical examination of ganders, 70 and 117 birds became diseased in the experimental and control group, respectively (Table II). The affected ganders were culled and replaced by healthy birds. From that time up to the end of the experiment, 46 (13.53%) and 97 (36.32%) of the ganders became affected in the experimental and control group, respectively. There was a statistically significant difference between the two groups in the number of affected animals.

After dividing the flock into two groups and treating the experimental group, from post-treatment day 12 up to the conclusion of the experiment the infertility rate of eggs collected in the treated group was significantly lower than that of the eggs collected in the control group during the same period (Table III). Further, the hatching rate of eggs collected from the experimental group was significantly higher as compared to the control group. Egg production per layer was also higher in the treated group.

A large proportion of phallic lymph samples collected on post-treatment days 14 and 50 yielded mycoplasma isolates. No statistically significant difference existed between the experimental and control samples in the rate of mycoplasma isolation; however, in the treated group a marked decrease (from 75.0 to 40%) was observed in the mycoplasma-positivity of the samples taken on post-treatment day 50, as compared to that of samples taken on post-treatment day 14 (Table IV).

Experiment II

Dynamutilin treatment also led to a pronounced decrease in the number of affected ganders in the medicated group. By the end of the experiment 34.91% and 71.02% of the ganders became affected with inflammation of the

Table II

Effects of Lincospectin and Tylan treatment in the goose flock affected with inflammation of the phallus

	Ganders		Layers	
	Experimental	Control	Experimental	Control
3 March				
Number of birds at the start of experiment	320	320	1066	1245
Sex ratio			3.33	3.89
20 April				
Clinical examination Affected	70 (21.88%)	117 (36.56%)	N. E.	N. E.
	$P < 0.01$			
Replacement	117	64	—	—
Sex ratio			2.90	4.66
1 June				
Clinical examination Affected	46 (13.53%)	97 (36.32%)	N. E.	N. E.
	$P < 0.01$			
Total:				
Total number of birds	437	384	1066	1245
Sex ratio			2.44	3.24
Total number of affected birds	116 (26.54%)	214 (55.23%)	N. E.	N. E.
	$P < 0.001$			

N. E.: Not examined

phallus in the treated and control group, respectively (Table V). No difference could be observed between the two groups in the infertility rate and hatching rate of eggs. At the same time, egg production of the treated group exceeded that of the control group by 0.79 egg per layer; the difference was significant ($P < 0.05$).

Experiment III

Similarly as in earlier experiments, egg production of the treated group increased after Dynamutilin and Lincospectin treatment. In 3 of the 16 egg-collection periods (26 February, 21 and 28 March) the number of eggs per layer was lower in the treated than in the control group; in all other egg-collection periods, however, it was higher (in most cases significantly higher) in the treated group as compared to the control. Thus, also egg production cal-

Table III

Effect of Lincospectin and Tylan treatment on egg production, infertility rate and hatching rate of eggs in goose flocks affected with inflammation of the phallus

Start of incubation	Infertility (%)			Hatching rate (%)			Egg production (n/layer)		
	Experi-mental	Control	χ^2 $P < 0.01$	Experi-mental	Control	χ^2 $P < 0.01$	Experi-mental	Control	χ^2 $P < 0.05$
5 April	38.6	38.7	N.S.	54.5	53.9	N.S.	3.33	2.54	
12 April	36.2	43.6		55.6	48.9		3.37	3.61	
20 April	33.3	35.5	N.S.	58.9	54.9		2.43	2.05	
27 April	27.0	32.5		65.4	59.2		1.94	1.80	
5 May	24.0	35.7		68.4	55.6		2.19	1.22	
11 May	23.3	37.8		69.4	54.6		2.16	1.87	
17 May	26.8	38.9		65.8	52.7		2.02	1.73	
25 May	35.4	47.8		56.1	45.7		2.19	1.80	
5 June	34.0	48.0		56.0	43.8		1.51	1.23	
Mean (for the whole production cycle)	30.8	38.6	$P < 0.01$	58.6	50.7	$P < 0.01$	21.24	18.75	$P < 0.01$

N.S. = not significant

Table IV

Results of mycoplasma isolation attempts from the phallic lymph of Lincospectin- and Tylan-treated and control ganders

Time of examination	Group	Number of birds examined	Positive	Negative	χ^2
On day 14 after the beginning of treatment	Control	19	12 (63.16%)	7	
	Experimental	20	15 (75%)	5	$P < 0.05$ (Experimental, day 14 vs. day 50)
On day 50 after the beginning of treatment	Control	14	9 (64.2%)	5	
	Experimental	15	6 (40.0%)	9	

culated for the whole egg-collection period was higher in the treated group (Table VI). In addition, in most of the incubation cycles, eggs of the treated group had a significantly lower infertility rate and a higher hatching rate than those collected in the control group. These parameters were more favourable in the treated group also when related to the whole production cycle.

Due to the low number of the samples examined, the results of mycoplasma isolation and serological examinations were monitored, using cumulative data for the entire period of experiment. There was no difference between the treated and control group in mycoplasma-isolation results obtained before the beginning of egg production and before treatment (in January and February,

Table V

Effect of Dynamutilin treatment on inflammation of the phallus in ganders

	Ganders		Layers	
	Experimental	Control	Experimental	Control
10 August				
Number of birds at the start of the experiment	385	418	1096	1575
Sex ratio			2.85	3.76
12 September				
Clinical examination	40	127	N.E.	N.E.
Affected	(10.39%)	(38.38%)		
	$P < 0.001$			
Replacement	—	127		
Sex ratio			3.18	3.76
10 October				
Clinical examination	24	98	N.E.	N.E.
Affected	(6.23%)	(23.44%)		
	$P < 0.001$			
Replacement	39	128		
Sex ratio			3.04	3.51
18 November				
Clinical examination	27	122	N.E.	N.E.
Affected	(7.01%)	(29.19%)		
	$P < 0.001$			
Sex ratio			3.29	4.83
19 December				
Clinical examination	38	131	N.E.	N.E.
Affected	(9.87%)	(31.32%)		
	$P < 0.001$			
Sex ratio			3.71	8.08
Total:				
Total number of birds	424	673		
Sex ratio	2.58	2.34		
Total number of diseased brids	148 (34.91%)	478 (71.02%)		
	$P < 0.001$			

N.E. = not examined

respectively). From March onwards mycoplasmas were isolated on significantly fewer occasions in the treated than in the control group (Table VII).

The indirect haemagglutination test, using antigens prepared from the two *Mycoplasma* strains, demonstrated antimycoplasma antibodies in the sera of birds. In December and January the number of positive birds was low, then, parallel with the progression of the production cycle, the rate of seropositivity increased. Particularly the number of birds positive to strain M. sp.

Table VI

Effect of Dynamutilin and Lincospectin treatment on egg production, infertility rate and hatching rate of eggs in goose flocks affected with inflammation of the phallus

Start of incubation	Infertility (%)			Hatching rate (%)			Egg production (n/layer)		
	Experimental	Control	χ^2 $P < 0.05$	Experimental	Control	χ^2 $P < 0.01$	Experimental	Control	χ^2 $P < 0.01$
9 February	18.4	21.5	N.S.	47.1	44.4	N.S.	0.96	0.46	
22 February	21.1	27.0		67.1	42.9		1.31	0.96	
26 February	18.7	20.1	N.S.	60.9	52.7		0.96	1.15	
29 February	17.8	17.7	N.S.	62.1	54.9		1.22	0.86	
4 March	20.4	13.3		66.2	53.4		1.22	0.69	
7 March	17.6	7.8		67.2	56.2		1.31	0.86	
11 March	5.6	21.2		64.1	64.3	N.S.	1.91	0.80	
14 March	9.5	16.0		72.3	52.4		1.31	0.86	
18 March	11.3	17.2		75.0	58.7		1.83	0.86	
21 March	11.7	13.6	N.S.	73.5	67.6		1.22	0.86	
25 March	15.6	14.1	N.S.	69.4	70.9	N.S.	0.35	1.03	
28 March	12.3	14.1	N.S.	74.8	71.6	N.S.	0.70	0.46	
4 April	12.0	13.6		67.3	65.8	N.S.	1.31	0.80	
11 April	12.6	16.2		70.9	65.0		1.65	0.63	
18 April	11.5	14.3		54.2	64.8		0.96	0.80	N.S.
25 April	13.0	14.0	N.S.	74.0	73.8	N.S.	0.96	0.69	
Mean	13.8	16.3	$P < 0.001$	67.1	60.1	$P < 0.001$	16.50	13.00	$P < 0.001$

N.S. = not significant

Table VII

Effect of Dynamutilin and Lincospectin treatment on mycoplasma isolation

Months of examination	Experimental		Control		χ^2
	Examined	Positive	Examined	Positive	
January	19	10	20	14	N.S.
January–February	39	27	38	32	N.S.
January–March	57	36	56	45	$P < 0.05$
January–April	75	47	72	55	$P < 0.05$

N.S. = not significant

1220 was high, while much fewer birds showed seroconversion to strain M. sp. 1221. In the initial period of production there was no difference between the treated and control group in the number of birds responding to mycoplasma antigen. On the other hand, later on the number of positively-reacting birds was significantly lower in the medicated group than in the control (Table VIII).

Experiment IV

Treatment of geese with Lincospectin reduced the incidence of the disease to half of that found for the control group (19.89 and 37.75%, respectively; Table IX). Egg infertility rate was significantly lower, while hatching rate significantly higher, in the experimental group than in the control. This difference had existed already at the start of the experiment.

Experiment V

In the experimental group treated with Tiamutin only 44 birds (7.89%) showed symptoms indicative of inflammation of the phallus, while in the control group 188 birds (35.27%) were affected. The difference is significant ($P < 0.05$). Egg production showed fluctuations between the starting dates of consecutive incubations. Periods in which egg production was higher in the experimental than in the control group occurred with approximately the same frequency as

Table VIII

Effect of Dynamutilin + Lincospectin treatment on the seroconversion of geese

Months of examination	Isolated strain	Experimental		Control		χ^2
		Tested	Positive	Tested	Positive	
December–January	1221	N.T.		N.T.		
	1220	40	21	40	23	N.S.
December–February	1221	59	4	58	12	$P < 0.05$
	1220	59	39	58	40	N.S.
December–March	1221	77	5	76	20	$P < 0.001$
	1220	77	40	76	54	$P < 0.05$
December–April	1221	95	10	92	24	$P < 0.01$
	1220	95	51	92	63	$P < 0.05$
December–May	1221	108	13	107	29	$P < 0.01$
	1220	108	55	107	72	$P < 0.05$

N.T. = not tested
N.S. = not significant

Table IX

Effect of Lincospectin against inflammation of the phallus in ganders

	Ganders		Layers	
	Experimental	Control	Experimental	Control
10 August				
Number of birds at the start of the experiment	414	398	1034	1566
Sex ratio			2.50	3.93
12 September				
Clinically affected	40 (9.66%)	127 (31.91%)	N.E.	N.E.
	$P < 0.001$			
Replacement	62	136		
Sex ratio			2.62	3.41
10 October				
Clinically affected	30 (6.88%)	98 (24.08%)		
	$P < 0.001$			
Sex ratio			3.01	4.22
Total:				
Total no. of birds	352	596	1034	1566
Sex ratio			2.94	2.63
Total no. of diseased birds	70 (19.89%)	225 (37.75%)		
	$P < 0.01$			

those in which the control group had higher egg production. However, taking into consideration the whole egg production period, the number of eggs per layer was 28.20 in the control and 29.25 in the experimental group. Evaluating these data by the χ^2 test, a significant difference was established in favour of the treated group.

The infertility rate of eggs was lower in the treated than in the control group in almost all egg-collection periods (Table X). Thus, the infertility rate calculated for the whole egg-production period was significantly lower in the treated than in the control group. At the same time, except for two batches of eggs, in the treated group the hatching rate of eggs was significantly higher than in the control. This was reflected also in the data calculated for the whole production cycle.

Table X

Effect of Tiamutin treatment on egg production, infertility rate and hatching rate of eggs in goose flocks affected with inflammation of the phallus

Start of incubation	Infertility rate (%)			Hatching rate (%)			Egg production (n/layer)		
	Experi-mental	Control	χ^2 $P < 0.001$	Experi-mental	Control	χ^2 $P < 0.001$	Experi-mental	Control	χ^2 $P < 0.001$
16 February	14.4	18.0		75.4	73.9	N.S.	N.E.	N.E.	
23 February	13.6	17.7		74.5	73.2	N.S.	1.12	1.44	
27 February	11.4	14.6		78.1	75.1		1.50	1.87	
3 March	10.4	13.8		79.7	76.7		1.36	1.36	N.S.
6 March	10.5	10.5	N.S.	77.5	77.0	N.S.	1.14	0.98	
10 March	10.5	10.5	N.S.	77.5	77.0	N.S.	1.14	0.98	
13 March	8.8	13.2		77.1	77.0	N.S.	1.09	0.77	
16 March	8.8	13.2		77.1	74.0		1.09	1.21	
17 March	9.8	13.4		76.9	74.7	N.S.	0.73	0.76	N.S.
20 March	10.9	13.9		79.2	74.4		0.93	1.23	
24 March	9.8	12.2		77.8	76.8	N.S.	1.51	1.02	
26 March	10.4	13.5		76.9	71.5		1.21	1.64	
31 March	9.4	14.1		77.6	73.8		1.37	1.64	
3 April	10.2	13.7		79.2	75.4		1.11	1.03	
6 April	7.1	15.5		79.8	72.0		0.82	0.72	
7 April	11.1	12.7		78.4	76.0	N.S.	0.73	0.67	
13 April	9.3	15.7		75.0	72.3		1.36	1.44	
14 April	11.0	15.2		75.8	70.3		0.78	1.14	
17 April	10.5	15.8		78.0	75.1		1.11	1.08	N.S.
21 April	10.2	14.0		74.8	68.8		1.29	0.94	
24 April	9.2	15.9		78.5	72.2		0.82	0.92	
28 April	16.6	16.4	N.S.	72.5	74.6	N.S.	1.17	1.13	N.S.
1 May	10.9	19.6		75.2	67.5		0.95	1.01	
8 May	16.5	21.5		N.E.	N.E.		1.87	1.71	
15 May	16.9	24.8		N.E.	N.E.		1.05	0.96	
21 May	14.7	28.5		N.E.	N.E.		0.43	0.25	
22 May	22.5	29.6		N.E.	N.E.		0.86	0.07	N.S.
28 May	N.E.	N.E.		N.E.	N.E.		0.55	0.36	
Mean	11.4	17.07	$P < 0.001$	78.16	76.77	$P < 0.001$	29.25	28.20	$P < 0.001$

N.E. = not examined, N.S. = not significant

Discussion

As indicated by the presented data, in the goose flocks treated with the antimycoplasma drugs applied, the incidence of inflammation of the phallus of ganders decreased by some 50%, a fact proving the efficacy of the said antibiotics against the disease. This efficacy was presumably due to reduction of the mycoplasma infection rate, reflected by results of mycoplasma isolation and serological examinations performed simultaneously with the treatments. Treatment resulted in a decreased rate of mycoplasma isolation from phallic lymph samples and a simultaneous drop in the number of birds seroconverted to mycoplasma in the treated group. However, in accordance with experiences

gained in other species of animals, treatment failed to result in a final eradication of mycoplasma infection. The failure might have been due to the fact that the applied antibiotics did not reach sufficient concentration in the phallic lymph or, for lack of experience, the applied doses were suboptimal or the route of application inadequate. Antibiotic treatment results in a decreased rate of clinically apparent disease and reduced incidence of mycoplasma infection; however, it does not completely eradicate inflammation of the phallus and mycoplasma infection from the flock. The results of medication trials furnish indirect evidence of an aetiological role of mycoplasmas in inflammation of the phallus of geese.

In earlier studies (Stipkovits et al., 1984), mycoplasma infection of geese was confirmed to have an aetiological role in airsacculitis and peritonitis, infertility of eggs, and early embryonic mortality; furthermore, its vertical spread via the egg was established. In the present experiments, treatment with the applied antimycoplasma drugs resulted in a decreased infertility rate and an increased hatching rate, suggesting that the beneficial effect of these drugs might be due to their efficacy against mycoplasmas. The results support the hypothesis that inflammation of the phallus and the related increase in egg infertility and reduction in egg production can be attributed to mycoplasma infection. This is substantiated by our observations according to which mycoplasmas could be isolated also from eggs collected in other goose flocks affected with inflammation of the phallus.

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PHARMACOKINETICS OF AMPICILLIN AND AMOXYCILLIN IN *BUBALUS BUBALIS* FOLLOWING INTRAMUSCULAR ADMINISTRATION

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The pharmacokinetics of ampicillin and amoxycillin was studied in buffalo calves following a single intramuscular administration (10 mg/kg). The values of the half-life for the absorption phase of ampicillin (15.4 ± 1.8 min) and amoxycillin (21.8 ± 4.3 min) showed rapid absorption. The elimination half-life of ampicillin was 1.93 ± 0.28 h and that of amoxycillin was 2.42 ± 0.27 h. The apparent volumes of distribution of ampicillin and amoxycillin were 0.97 ± 0.10 and 0.68 ± 0.04 L/kg, respectively. The total body clearance, which is the sum of all clearance processes, was 1.8-fold higher for ampicillin than for amoxycillin. The bioavailability of ampicillin was 56 ± 2.9% and that of amoxycillin was 47 ± 4.4%. Twenty % of the ampicillin and 18% of the amoxycillin were bound to serum proteins at concentrations ranging from 2 to 20 μg/ml. On the basis of pharmacokinetic values, satisfactory intramuscular dosage regimens would be 13 mg ampicillin and 10 mg amoxycillin/kg body mass, which should be repeated at an interval of 6 and 8 h, respectively.

Keywords. Pharmacokinetics, ampicillin, amoxycillin, in vitro, serum protein binding, buffalo, calves.

Ampicillin and amoxycillin are valuable semisynthetic penicillins because of their clinical effectiveness against a wide range of bacterial infections in a variety of domestic animals (Bywater, 1982; Huber, 1982). Pharmacokinetics of ampicillin and amoxycillin has been investigated in cattle and sheep (Chaleva, 1977, 1981*a*; Ruthers et al., 1980; Carli et al., 1981; Palmer and Bywater, 1982; Stanton et al., 1982), horses (Pugh, 1977: Yeoman, 1977; Beech et al., 1979; Keefe et al., 1980) and swine (Chaleva, 1981*b*). However, such information on these antibiotics is not available in buffalo species. Pharmacokinetic studies of antimicrobials which provide a basis for determination of their optimal dosage regimens are relevant in animals in which the drugs are to be used clinically. Accordingly, the present study was undertaken to investigate the kinetic disposition and dosage of ampicillin and amoxycillin in *Bubalus bubalis* after a single intramuscular administration. The serum protein binding (in vitro) of ampicillin and amoxycillin was also determined.

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Materials and methods

Animals and treatment

Experiments were conducted on 8 healthy male buffalo calves weighing 80–123 kg. The animals were housed in departmental animal shed with concrete floor and were kept on green fodder of the season and wheat straw. The water was provided ad libitum. Ampicillin as ampicillin sodium (Inga Pharmaceuticals, Bombay) and amoxycillin as amoxycillin sodium (Beecham Labs, Bristol, Tenn) were each injected intramuscularly in a single dose of 10 mg/kg body mass to two groups of 4 animals each.

Experimental and assay procedures

Blood samples were collected by jugular venipuncture at 0, 15, 30, 45 min and 1, 1.5, 2, 3, 4, 6, 8, 12 and 24 h after the administration of antibiotics. The serum was separated soon after its release and was cleared by centrifugation. The concentrations of ampicillin and amoxycillin in serum samples were determined by a microbiological assay method using *Sarcina lutea* (ATCC 9341) as test organism (Arret et al., 1971).

The in vitro serum protein binding of ampicillin and amoxycillin at 2, 5, 10 and 20 μg/ml concentrations was determined by the equilibrium dialysis technique (Kunin et al., 1959; Kunin, 1965). Serum collected from 3 animals was pooled and 3 separate experiments were carried out for each concentration of drug.

Pharmacokinetic procedures

Serum drug concentrations plotted on semilogarithmic scale as a function of time were described by one-compartment open model. Serum concentration–time data were analysed separately for each experimental animal. The pharmacokinetic values were calculated by the method described by Gibaldi and Perrier (1975). Based on these data, optimal dosage regimens of ampicillin and amoxycillin for buffalo species were determined.

Results

Serum concentrations and serum protein binding

Table I shows the serum levels of ampicillin and amoxycillin at various time intervals after their single intramuscular administration (10 mg/kg). Appreciable levels of ampicillin (5.25 ± 0.17 μg/ml) and amoxycillin ($5.85 \pm \pm 0.53$ μg/ml) were detected in serum 15 min after injection. The peak serum

concentrations (C_{max}) of ampicillin (10.6 ± 1.02 μg/ml) and amoxycillin (10.2 ± 0.52 μg/ml) were detected at 30 min. Concentration of drugs in serum declined to less than 0.2 μg/ml at 6 h after treatment.

The results on in vitro serum protein binding of ampicillin and amoxycillin at different serum concentrations are shown in Table II. Ampicillin and amoxycillin were bound to the extent of 18.8 ± 1.4 to 21.4 ± 1.8 and 17.2 ± ± 0.8 to 19.1 ± 1.7% with overall mean of 20.4 and 18.1%, respectively.

Disposition kinetics

The values of kinetic parameters which describe disposition of drugs are presented in Table III. The half-life for absorption and elimination of ampicillin were 15.4 ± 1.8 min and 1.93 ± 0.28 h and the respective values for

Table I

Concentrations of ampicillin and amoxycillin in serum of buffalo calves after a single intramuscular injection of 10 mg/kg body mass

Time	Ampicillin	Amoxycillin
15 min	5.25±0.17	5.85±0.53
30 min	10.6 ±1.02	10.24±0.52
45 min	7.96±0.22	8.86±0.93
1 h	6.34±0.27	7.79±1.01
1.5 h	5.06±0.41	5.84±0.39
2 h	3.69±0.17	4.21±0.25
3 h	2.84±0.19	3.56±0.41
4 h	1.70±0.13	1.27±0.61
6 h	0.16±0.008	0.17±0.02
8 h	0.12±0.007	0.13±0.01
12 h	0.10±0.006	0.09±0.004

Values given are expressed as μg/ml and represent the mean ± SE of the results obtained from 4 animals

Table II

Ampicillin and amoxycillin protein binding (in vitro) in buffalo serum

Drug	Drug concentration (μg/ml)	Extent of binding in serum			(% bound)
		Experiment No.			Mean ± SE
		1	2	3	
Ampicillin	2	18.4	24.5	21.35	21.42±1.76
	5	19.5	23.25	20.45	21.07±0.92
	10	22.3	17.95	21.0	20.42±1.29
	20	19.0	21.5	16.0	18.83±1.38
Amoxycillin	2	21.25	19.3	14.5	18.35±2.01
	5	18.25	16.6	22.45	19.1 ±1.74
	10	13.0	17.65	22.75	17.8 ±2.82
	20	17.5	18.35	15.7	17.18±0.78

Table III

Disposition kinetics of ampicillin and amoxycillin in buffalo calves following a single intramuscular administration (10 mg/kg)

Parameter[a]	Unit	Ampicillin	Amoxycillin
Ka	min^{-1}	0.048±0.006	0.035±0.006
$t_{1/2Ka}$	min	15.40±1.85	21.81 ±4.32
B	$\mu g \cdot ml^{-1}$	7.53 ±0.58	7.73 ±0.70
β	h^{-1}	0.383±0.057	0.298±0.034
$t_{1/2\beta}$	h	1.93 ±0.28	2.42 ±0.27
Area	$\mu g \cdot ml^{-1} \times min$	1288±62.9	1421 ±93.5
$V_{d(area)}$	$L \cdot kg^{-1}$	0.97 ±0.10	0.68 ±0.04
Cl_B	$ml \cdot min^{-1} \cdot kg^{-1}$	6.14 ±1.08	3.41 ±0.53
F	%	56.49 ±2.86	47.32 ±4.39

Values given are the mean ± SE for 4 animals. [a] Kinetic parameters have been described by Gibaldi and Perrier (1975)

amoxycillin were 21.8 ± 4.3 min and 2.42 ± 0.27 h. The estimate for apparent volume of distribution which relates the drug concentration in serum to the total amount of drug in body after distribution equilibrium has been attained was higher for ampicillin (0.97 ± 0.10 L/kg) than for amoxycillin (0.68 ± 0.04 L/kg). On the basis of the area under the serum concentration–time curves and elimination rate constants of drugs after intramuscular and intravenous treatments, it was calculated that 56.5% of ampicillin and 47.3% of amoxycillin were absorbed from the intramuscular administration sites.

Discussion

The serum drug concentration–time profiles of ampicillin and amoxycillin indicated that both drugs are rapidly absorbed following intramuscular injection in buffalo species. This is further substantiated by the low values of absorption half-life of these antibiotics established in the present study. Relatively short elimination half-life of ampicillin (1.93 h) as compared to amoxycillin (2.42 h) indicated a slower elimination of the latter drug in buffaloes. The values of elimination half-life of ampicillin and amoxycillin established in the present study are 2.6–3.2-fold longer than that reported in cows. In cows, elimination half-life of these antibiotics has been reported as 45 min (Ziv and Nouws, 1979).

The higher value of apparent volume of distribution ($V_{d(area)}$) of ampicillin than amoxycillin suggests a better penetration of the former drug in the body of buffaloes. In comparison to ampicillin, the values of total body clearance of amoxycillin were lower due to its low values of elimination rate constant (β) and $V_{d(area)}$. For calculating the bioavailability, the values of the

total area under the curve (AUC) and β established in these animals following a single intravenous injection of ampicillin (10 mg/kg) and amoxycillin (10 mg/kg) 3 weeks prior to the present study were also employed. The fractions of ampicillin and amoxycillin absorbed or the bioavailability following intramuscular administration were calculated to be 56 and 47% ,respectively.

The in vitro protein binding studies revealed that ampicillin and amoxycillin were bound to buffalo serum to the extent of 20.4 and 18.1%, respectively. Similar serum protein binding of these antibiotics has been reported in bovines (Palmer and Bywater, 1982). Conversely, the binding of ampicillin with horse serum has been reported to be very low, ranging between 6.8 and 8% (Durr, 1976).

The pharmacokinetics and suitable dosage regimen of a drug are best determined in the animals and environment in which the drug is to be used clinically. The higher variation in pharmacokinetic behaviour of ampicillin and amoxycillin in buffaloes than that reported in cattle further necessitates the calculation of optimal dosage regimens of these antibiotics for buffaloes on the basis of kinetic values as established in the present study. Ampicillin and amoxycillin have an essentially identical antibacterial spectrum with only few exceptions and a concentration of 1.5 μg/ml is usually considered minimum inhibitory concentration (MIC) for both antibiotics (Mandell and Sande, 1980; Bywater, 1982). Accordingly, intramuscular dosage regimens based on the desirable minimum concentration of drugs in serum (1.5 μg/ml) have been calculated by the formula,

$$\text{Cp min} = \frac{\text{B}e^{-\beta t}}{1 - e^{-\beta t}}.$$

where Cp min is the minimum desirable concentration of the drug in serum, B is zero-time intercept of serum drug concentration and e represents the base of natural logarithm. β is elimination rate constant and t is the time. On this basis, satisfactory intramuscular dosage regimens would comprise 13 mg ampicillin and 10 mg amoxycillin/kg body mass repeated at an interval of 6 and 8 h, respectively.

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PHARMACOKINETICS AND BIOCHEMICAL EFFECTS OF DIACETYL MONOXIME IN *BUBALUS BUBALIS*

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Pharmacokinetics and biochemical effects of diacetyl monoxime (DAM) were investigated in male buffalo calves after single intravenous administration (15 mg/kg body mass). The time-course of plasma DAM concentrations was adequately described by a two-compartment open model. At 1 min of administration, the peak plasma DAM level was 53 ± 2 μg/ml. The elimination half-life, apparent volume of distribution and total body clearance were 4.4 ± 0.03 h, 0.76 ± 0.08 L/kg and 120 ± 14 ml/h/kg, respectively. DAM did not alter the activities of erythrocyte cholinesterase, plasma cholinesterase and serum acid phosphatase, and induced no apparent toxicity. DAM elevated the serum levels of carboxylesterase, aspartate aminotransferase and alanine aminotransferase, whereas it lowered the levels of serum lactate dehydrogenase. The altered serum enzyme activities returned to their control values within 12 h of administration of DAM.

Keywords. Diacetyl monoxime, pharmacokinetics, blood enzymes, plasma protein binding, buffalo, in vitro.

The use of oxime reactivators in conjunction with atropine constitutes an efficacious therapy against organophosphate poisoning in man and a variety of animal species (Clement, 1979; Bošković et al., 1980; Clarke et al., 1981). The therapeutic effect of oxime reactivators is dependent, among other factors, on their levels in blood and target organs. For computing the optimal therapeutic regimen, pharmacokinetics of different oxime reactivators have been investigated in man (Jager et al., 1958; Barkman et al., 1963, Sidell and Groff, 1970; 1971; Swartz and Sidell, 1974) and laboratory animals (Dultz et al., 1957; Das Gupta et al., 1979; Moorthy et al., 1981). However, such information on diacetyl monoxime (DAM), an oxime reactivator known to readily penetrate the blood–brain barrier and reactivate acetylcholinesterase enzyme in the central nervous system (Taylor 1980), is absolutely lacking in buffalo species. Recently DAM has been reported as an effective antidote against fenitrothion poisoning in buffaloes (Malik et al., 1984). In order to use DAM efficaciously and on a rational basis in buffalo species, we have investigated the pharmacokinetics of DAM in healthy male buffalo calves after a single intravenous administration. In addition, in vitro plasma protein binding and blood biochemical alterations induced by DAM were also studied.

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Materials and methods

Chemicals

DAM was purchased from Aldrich Chemical Co., Milwaukee, Wisconsin. Indophenyl acetate was obtained from Eastman Organic Chemicals, Rochester, New York. N-(1-Naphthyl) ethylene diammonium dichloride was commercially obtained from E. Merck AG, Darmstadt. All other chemicals of highest purity were purchased from E. Merck (India) Ltd., Glaxo Laboratories (India) Ltd. and Loba Chemie Indoaustranal Co., Bombay.

Animals

Healthy male buffalo calves weighing approximately 82 kg were purchased from the local market. All animals were quarantined for 2 weeks and subjected to regular clinical examination. The animals were kept in a departmental animal shed and were given standard ration and water *ad libitum.*

Dosage of DAM and collection of samples

DAM was administered in saline into one of the jugular veins at the dosage level of 15 mg/kg body mass. Blood samples were withdrawn from the opposite jugular vein into heparinized and non-heparinized tubes before, and at several times after, injection of oxime reactivator. Erythrocytes, plasma and serum were separated at room temperature.

Assay procedures

The levels of DAM in plasma were determined according to the method of Dultz et al. (1957). The cholinesterase (ChE) activity in erythrocytes and plasma was measured according to the method of Fleisher et al. (1955) as modified by Sharma et al. (1973). Serum carboxylesterase activity was determined by using indophenyl acetate as substrate (Mendoza et al., 1971). Aspartate aminotransferase, alanine aminotransferase, acid phosphatase and lactate dehydrogenase in serum were determined by the procedures described by Wootton (1965).

In vitro plasma protein binding of DAM at 2, 5, 10, 20 and 40 μg/ml concentrations was determined by the equilibrium dialysis technique (Kunin et al., 1959; Kunin, 1965).

Processing of data

The plasma DAM concentrations plotted on semilogarithmic scale as a function of time were analysed separately for each experimental animal. Various pharmacokinetic parameters were calculated by the method described

by Gibaldi and Perrier (1975). Mean value and standard error were calculated for each kinetic parameter.

Statistical analysis was performed by Student's *t* test. $P < 0.05$ was considered significant.

Results

Plasma levels of DAM plotted on a semilogarithmic scale as a function of time are shown in Fig. 1. The mean peak plasma DAM concentration of 53 ± 2 μg/ml was recorded at 1 min which rapidly declined to 22 ± 1 μg/ml at 10 min. Thereafter, DAM disappeared gradually from the plasma and was detected in traces (0.9 ± 0.2 μg/ml) at 1440 min. Evaluation of the results on observed plasma levels of DAM indicated that the data can be best fitted to a two-compartment open model and were adequately described by a biexponential equation, $Cp = Ae^{-\alpha t} + Be^{-\beta t}$, where Cp is the plasma concentration of DAM at time t, A and B are zero-time intercepts of initial and terminal phases of the concentration–time curve, α and β are distribution and elimination rate constants, respectively, and e represents the base of natural logarithm.

Various pharmacokinetic parameters estimated from the plasma concentrations of DAM after its intravenous administration are shown in Table I. The mean values of distribution half-life ($t_{1/2\alpha}$) and elimination half-life ($t_{1/2\beta}$) were 0.045 ± 0.006 and 4.39 ± 0.03 h, respectively. The apparent volume of distribution ($V_{d(area)}$) and total body clearance (Cl_B) varied to the extent of 0.55 to 1.01 l/kg and 87.5 to 162.6 ml/h/kg, respectively. The mean ratio of distribution of drug between tissue and plasma was 2.01 ± 0.1.

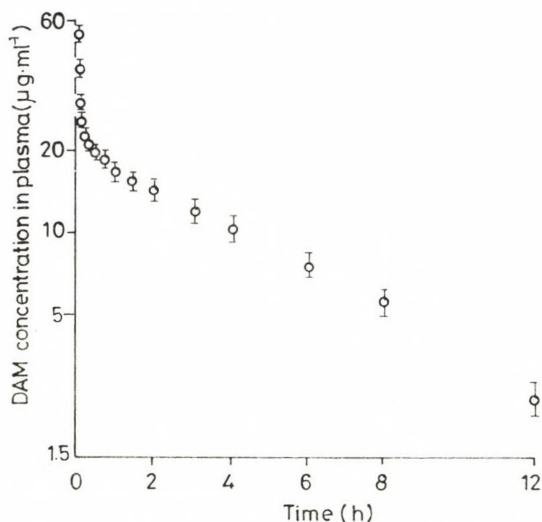

Fig. 1. Plasma concentrations profile of DAM after a single intravenous dose of 15 mg/kg body mass to buffalo calves. Values are presented as mean ± SE of 4 animals

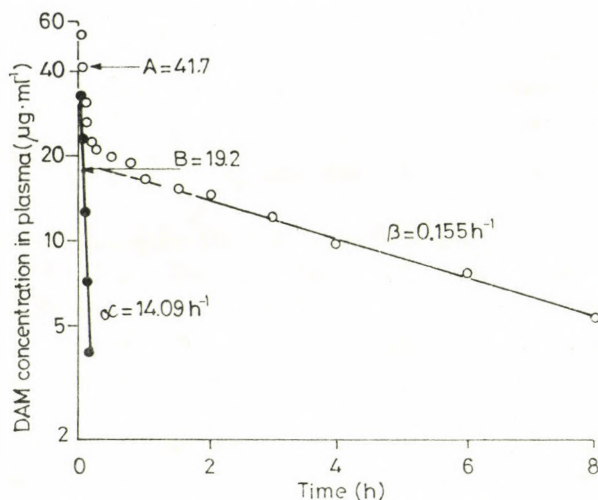

Fig. 2. Plasma DAM concentrations in a representative buffalo calf given a single intravenous dose of 15 mg/kg body mass. The calculated points in the distribution phase (●) were obtained by the feathering technique

Figure 2 shows a semilogarithmic plot of plasma levels of DAM vs time for a representative animal. The coefficients A (41.7 μg/ml) and B (19.2 μg/ml) and the rate constants α (14.09 h^{-1}) and β (0.155 h^{-1}) calculated are also presented. The data of this animal can be best described by the following equation:

$$Cp = 41.7e^{-14.09t} + 19.2e^{-0.155t}$$

Table I

Pharmacokinetic parameters of DAM following a single intravenous administration (15 mg/kg body mass) in buffalo calves

Parameter[a]	Unit	Mean ± SE[b]	Value for a representative animal
Cp°	$\mu g \cdot ml^{-1}$	61.4±5.12	60.9
A	$\mu g \cdot ml^{-1}$	41.3±2.99	41.7
B	$\mu g \cdot ml^{-1}$	20.3±2.2	19.2
α	h^{-1}	14.28±1.27	14.09
β	h^{-1}	0.158±0.001	0.155
$t_{1/2\alpha}$	h	0.045±0.006	0.049
$t_{1/2\beta}$	h	4.39±0.03	4.46
K_{12}	h^{-1}	10.56±1.28	9.22
K_{21}	h^{-1}	5.59±0.94	4.55
K_{el}	h^{-1}	0.476±0.016	0.48
$V_{d(area)}$	$L \cdot Kg^{-1}$	0.76±0.08	0.76
AUC	$\mu g \cdot ml^{-1} \cdot h$	130.9±13.8	126.8
Cl_B	$ml \cdot h^{-1} \cdot kg^{-1}$	120.0±13.6	118.3
T/p	ratio	2.01±0.10	2.1

[a]Pharmacokinetic parameters described by Gibaldi and Perrier (1975); [b]Values are from 4 animals.

In vitro plasma protein binding of DAM

The extent of in vitro binding of different concentrations of DAM to plasma proteins of buffalo calves is given in Table II. DAM at concentrations ranging from 2 to 40 μg/ml of plasma was bound to the extent of 52–59% with an overall mean value of 56%.

Table II

In vitro binding of DAM to plasma proteins of buffalo calves

Experiment No.	Concentration of DAM ($\mu g \cdot ml^{-1}$)				
	2	5	10	20	40
1	50.5	55.5	49.3	55.6	52.9
2	50.5	77.7	77.7	56.1	54.7
3	53.5	50.6	51.1	55.7	51.5
4	51.6	53.8	57.7	54.6	56.8
Mean ± S.E.	51.5 ± 0.71	59.4 ± 6.2	58.9 ± 6.5	55.5 ± 0.33	53.9 ± 1.1

Values given are expressed as percentage of drug bound with plasma proteins

Table III

Effect of a single intravenous administration of DAM (15 mg/kg body mass) on the blood enzymes of buffalo calves

Enzyme	Time after DAM administration (min)					
	0	30	60	120	240	720
Erythrocyte ChE	1584±25	1591±39	1622±21	1558±39	1581±14	1588±22
Plasma ChE	190±11	195±12	195±9.0	196±9.4	186±5.8	205±8.2
Serum carboxyl-esterase	125±3.1	140±1.2[a]	155±4.9[a]	150±4.4[a]	150±4.4[a]	135±6.0
Serum aspartate aminotransferase	62±1.9	66±1.6	70±2.7[b]	81±2.9[a]	73±1.4[a]	64±1.1
Serum alanine aminotransferase	59±2.2	66±4.6	72±3.6[b]	74±4.2[b]	74±4.8[b]	67±5.5
Serum lactate dehydrogenase	299±10	258±20	251±15[b]	245±10[b]	243±10[a]	280±20
Serum acid phosphatase	4.9±0.09	5.0±0.06	5.1±0.15	5.0±0.17	5.2±0.27	5.0±0.30

Values given for erythrocyte ChE and plasma ChE (nmol acetylcholine hydrolysed/min/ml), serum carboxylesterase (nmol indophenol formed/min/ml), serum aspartate aminotransferase and serum alanine aminotransferase (nmol pyruvate formed/min/ml), serum lactate dehydrogenase (nmol pyruvate reduced/min/ml) and serum acid phosphatase (nmol phenol liberated/min/ml) are mean ± SE of the results obtained from 4 animals.

[a] Statistically significant ($P < 0.01$) difference when compared with 0-min value.

[b] Statistically significant ($P < 0.05$) difference when compared with 0-min value.

DAM-induced biochemical alterations

The effects of a single intravenous administration of DAM (15 mg/kg body mass) on levels of various blood enzymes are summarized in Table III. DAM did not alter the activities of erythrocyte ChE, plasma ChE and serum acid phosphatase. It produced significant elevation in the serum levels of carboxylesterase, aspartate aminotransferase, alanine aminotransferase and lowered the levels of serum lactate dehydrogenase. DAM at the given dosage induced no apparent toxicity. The altered activities of various serum enzymes returned to their control values within 12 h of administration of oxime reactivator.

Discussion

To characterize the pharmacokinetics of DAM in healthy male buffalo calves, DAM was administered in a dose of 15 mg/kg body mass by the intravenous route. The dosage level of DAM employed in the present study is quite comparable to the doses given for treatment of organophosphate poisoning in man and animals (Jager et al., 1958; Wright et al., 1966; Taylor, 1980).

DAM exhibited distinct distribution and elimination phases (Figs 1 and 2) and hence data were adequately described by a two-compartment open model. The high value of rate constant α (14.28 h^{-1}) and the comparatively low value of β (0.158 h^{-1}) indicated that DAM was rapidly distributed in the body and then slowly eliminated. The elimination half-life ($t_{1/2\beta}$) of DAM in buffalo calves (4.39 h) established in this study was similar to the $t_{1/2\beta}$ of DAM for dogs (4.5 h) but was 2-fold longer than that reported for rats (Dultz et al., 1957). On the other hand, a relatively longer $t_{1/2\beta}$ of DAM (7.4 h) has been reported in man (Jager et al., 1958).

The calculated value of 0.76 L/kg of $V_{d(area)}$ and the high tissue/plasma ratio of 2.01 indicated a good penetration of the drug into body tissues. The relatively low value (0.476 h^{-1}) of elimination rate constant (K_{el}) suggested that the drug was slowly eliminated from central compartment. This fact was further substantiated by the low value of Cl_B of DAM (120 ml/h/kg) in buffalo calves.

The moderate in vitro plasma protein binding of DAM observed in the present study suggests that a good percentage of the drug is available in circulation to penetrate into site of action. Low to moderate extents of protein binding ($< 80\%$) have relatively little influence on the distribution and elimination of drugs, since the drug–albumin interaction is readily reversible (Baggot, 1982). Following systemic administration, DAM has been reported to penetrate the blood–brain barrier of buffalo calves in appreciable quantities (Srivastava et al., 1985).

It is apparent from data shown in Table III that administration of DAM did not alter the levels of erythrocyte ChE, plasma ChE and serum acid phosphatase enzymes. However, DAM significantly elevated the levels of the serum carboxylesterase enzyme. Such an effect of DAM may be of additive therapeutic value as it is likely to enhance the detoxification of circulating organophosphorus insecticides that are selectively bio-inactivated by carboxylesterases. A widely-used organophosphorus insecticide, malathion, is largely metabolized by carboxylesterase enzyme in mammals (Talcott et al., 1979; Mallipudi et al., 1980; Malik and Summer, 1982). DAM also increased the serum levels of aspartate aminotransferase and alanine aminotransferase but lowered the levels of serum lactate dehydrogenase. The latter effect of DAM may antagonize the elevation in levels of serum lactate dehydrogenase induced by organophosphorus insecticides in poisoned animals (Malik et al., 1984). Results of the present study suggest that DAM, given in the dose of 15 mg/kg body mass intravenously, may be employed safely in buffalo species.

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FAILURE TO DEMONSTRATE INTESTINAL ABSORPTION OF RNA IN THE NEWBORN PIG PRELIMINARY COMMUNICATION

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The authors failed to demonstrate absorption of orally-administered RNA in one-week-old piglets which were already over the colostral absorptive period. Neither could they find absorption of RNA in newborn piglets after oral or intraintestinal administration. Possible explanations of the findings are discussed.

Keywords. Pig, intestinal absorption, RNA, nucleic acid, newborn.

The gut of pig and other ungulate species absorbs colostral proteins non-selectively on the first one or two days of life. Thus the piglet, born agammaglobulinaemic, acquires antibodies, essential for extrauterine life, from the maternal colostrum (Brambell, 1970; Baintner, 1986). Heterologous proteins and soluble non-protein macromolecules, e.g. polyvinylpyrrolidone, are absorbed as well, especially in colostrum-fed piglets (Clarke and Hardy, 1971). Absorption is mediated by the apical canalicular system (Veress and Baintner, 1971) and by large eosinophilic vacuoles (Comline et al., 1951) without involvement of immunoglobulin receptors (Baintner and Kocsis, 1984), as opposed to the receptor-mediated, selective IgG absorption in the small intestine of the suckling rat (Jones and Waldmann, 1972).

We could not find data about the possible ability of the intestine of newborn ungulates to absorb nucleic acids, one of the most important groups of macromolecules. We tested, therefore, the absorption of RNA to elucidate further the mechanism of macromolecular absorption. Our attempts at demonstrating RNA in piglets' serum after feeding RNA mixed with colostrum failed, however.

Materials and methods

Fourteen half-day-old and two one-week-old piglets of Large White x Landrace breed, originating from five litters, were used.

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Piglets born at night were removed from the sow in the morning and were starved for 2 h before use. Half g/kg body mass RNA was administered to two piglets through gastric tube. The experiment was repeated twice. Two other piglets in ether narcosis were injected with 0.25 g RNA into a surgically exposed, 25 cm long, ligated jejunal loop, then the abdominal wall was closed with sutures. Two other piglets were injected into the heart with 0.2 g RNA.

Before administration, 0.5 g yeast RNA (Merck) was dissolved in 10 ml 0.1 M sodium acetate and mixed with 10 ml bovine colostrum to promote absorption of macromolecules. For intracardiac administration colostrum-free RNA solution was used.

Each trial included a control piglet, which was provided with an RNA-free mixture of the solvents. Both the control and the experimental piglets were kept isolated from the sow for 4 h to allow time for absorption, thereafter, a blood sample was taken by cardiac puncture in ether narcosis.

Extraction and detection of RNA: two ml serum was mixed with 2 ml acetate buffer of pH 5 and 2 ml phenol, the mixture was kept at 60 °C for 10 min, then cooled to 4 °C and centrifuged (5000 *g*, 5 min). Four ml of the upper phase was removed, 1 ml distilled water and 0.4 ml lanthanum solution (1 g lanthanum nitrate in 100 ml of 1.5% perchloric acid) were added (Girard, 1967). The tubes were kept at 4 °C overnight, then centrifuged; the supernate was discarded, and the precipitate dissolved in 0.2 ml tris-HCL buffer. The solution was examined by both horizontal and vertical agarose gel electrophoresis according to Meyers et al. (1976).

The slabs were stained with 50 μg/ml ethidium bromide solution and viewed under a shortwave UV transilluminator.

Results and discussion

As previously expected, we failed to demonstrate absorption of orally-administered RNA in one-week-old piglets which were already over the colostral absorptive period. Neither could we find absorption of RNA in newborn piglets after oral or intraintestinal administration. Although small amounts of material were precipitated by the lanthanum solution overnight from the phenol-treated sera of most of the piglets, the dissolved precipitate did not fluoresce with ethidium bromide on the agarose slabs. The newborn piglets used for the experiment were able to transmit colostral proteins as demonstrated by the presence of alimentary proteinuria (Baintner, 1970).

One possible explanation for the findings is that the newborn piglets were unable to absorb RNA. However, there are alternative explanations as well: small quantities of absorbed RNA may be degraded by plasma RNases or remain below the threshold of detection. The RNA injected by the intra-

cardiac route did not disappear from the piglets' circulation rapidly. Intestinal and serum RNase values of piglets will be published later.

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SOME NORMAL PHYSIOLOGICAL VALUES IN BLOOD SAMPLES OF CHICKEN EMBRYOS SHORT COMMUNICATION

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Plasma aspartate aminotransferase (AST), glucose, total cholesterol, total protein and albumin/globulin ratio were determined in chicken embryos on the 19th day of incubation. The results may serve as reference values, previously missing in the literature.

Keywords. Blood plasma parameters, chicken, embryology.

The use of birds as test models in experimental and environmental toxicology seems to get more and more importance. Although an emphasis on human health is clearly appropriate, it must be remembered that humans are an intricate part of the biosphere and as such are subject to various facets of environmental quality that affect other life forms.

Avian models have been favoured by this kind of toxicological research because birds are abundant, highly visible, have diverse behaviours and habitat associations, and can easily be studied in both the laboratory and natural environment. Chicken eggs are inexpensive, available in virtually unlimited supply, and can be used in large numbers with limited staff, equipment, and space.

The present paper attempts to help those undertaking embryological and teratological studies on avian embryos, including studies on changes occurring in certain serum parameters.

Materials and methods

Fertile chicken (Shaver Starcross 288) eggs were used. Blood samples were withdrawn from chicken embryos on the 19th day of incubation. For this purpose, eggs were candled to determine the position of the chorioallantoic vessels and a small opening was made. An exposed chorioallantoic vein was nicked and a heparinized capillary tube was filled ((Hoffman and Ramm, 1972; Várnagy, 1981). After centrifugation and separation of blood plasma glucose concentration was measured in a glucose analysis system (Zender, 1963). The total plasma cholesterol (Watson, 1960), plasma AST (Kar-

men, 1955) and plasma total protein (Chromy and Fischer, 1977) were measured by spectrophotometric assays. The albumin/globulin ratio was estimated on the basis of polyacrylamide gel analysis (Man and Whitehead, 1968).

Results

A total of 414 plasma samples obtained from chicken embryos were examined each for one of five parameters. Detailed data are given in Table I.

Table I

Some plasma parameters of chicken embryos ($\bar{x} \pm$ S.D.)

Parameter	$\bar{x} \pm$ S.D.	n
AST (U/l)	23.50±3.73	n = 90
Glucose (mmol/l)	10.67±1.30	n = 90
Cholesterol (mmol/l)	11.07±1.18	n = 90
Total protein (g/l)	24.03±1.27	n = 90
Albumin/globulin	0.71±0.03	n = 54

Discussion

In the period preceding hatch-out the blood vessels of the chicken embryo are well developed (Romanoff, 1960). From plasma samples collected in heparinized glass capillaries, certain parameters can be determined by simple spectrophotometric methods because the blood volume obtainable by this method is 500–800 μl/egg. Most of the studies on avian embryology and teratology report only morphological data (Várnagy, 1980; Jelinek, 1982; Hoffman and Albers, 1984) and the plasma parameters are neglected. Plasma AST levels were investigated by Hoffman and Sileo (1984) for mallard *(Anas platyrhynchos)* embryos. The average AST level for 18 days old mallard embryos was found to be 31 U/l. For 15–35 days old chicken Prosser (1973) reported the following data: glucose, 7–14 mmol/l; total protein 32 g/l; albumin/globulin, 0.90; total cholesterol, 4.8–6.2 mmol/l. A comparison of these data to the data obtained for the final embryonic stage (see Table I) points to the way of normal physiological development.

By describing the experimental data the present paper attempts to be of use for research workers interested in avian embryology.

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DYNAMIC CHANGES IN OVARIAN STRUCTURES AND IN PROGESTERONE SECRETION IN THE COW THROUGHOUT THE CALVING-TO-CONCEPTION INTERVAL*

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The development of ovarian structures was studied in 39 cows by rectal palpation throughout the calving-to-conception interval. The accuracy of determining those structures was examined by running simultaneous assay of progesterone in milk. A much higher incidence of large follicles was observed in the last days of secretory cycles in the cows conceiving early (up to 92 days post partum) than in those conceiving late. Highest accuracy of rectal examinations was connected with the presence of corpus luteum, or with both ovaries smooth early post partum. The lowest accuracy was connected with the absence of corpus luteum.

Keywords. Ovary, follicle, rectal palpation, cow, progesterone, calving-to-conception interval.

The postpartum period is the most important phase of the reproduction cycle in the cow. Monitoring that period is essential for increasing reproductive efficiency by reducing losses due to fertility disorders. Although much analytical progress has been made in recent years, the clinical examination of the cow, and especially rectal palpation, is still the most popular way of evaluating reproductive fitness of the animal. However, rectal palpation results may not reflect the functional state of the ovaries, due to the difficulties in determining ovarian structures (Dawson, 1975).

The aim of this study was to monitor changes in ovarian morphology as determined by rectal palpation, and changes in ovarian secretory activity as reflected by progesterone measurements in milk. Confronting those two sets of results enabled us to evaluate the accuracy of rectal palpations in the cow throughout the entire calving-to-conception interval.

Materials and methods

From a large group of primiparous cows of Polish lowland breed (Friesian) 39, all in good condition, were selected which had shown no disorders and developed no endometritis post partum. During winter the animals were fed

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The data presented here were partly used in the thesis presented by the junior author.

silage, hay and concentrates with mineral supplementation; during summer they were grazing and supplemented with minerals. They were milked twice daily. The mean yield for the first 100 days of lactation was 1411 l. The studies were carried out in the years 1982–1983. Rectal palpations were started in the first week after calving and were continued once weekly. The following structures were discerned: smooth, undifferentiated ovary (U), small, usually multiple, follicles, up to 10 mm in diameter (S); large ovarian follicles (L) estimated as being larger than 10 mm; corpora lutea (C) — structures characteristic by palpation, discernible from thick-walled cysts.

The cows were watched twice daily for oestrous behaviour. They were inseminated at appropriate times by using semen frozen in "straws", prepared by the local insemination centre.

Progesterone was measured in whole, unextracted milk by a radioimmunoassay technique employing a ^{125}-labelled derivative (Stupnicki and Kula, 1980). Milk was sampled twice weekly. Concentrations of progesterone $\leq$ 2 ng/ml were considered to be zero, and to represent lack of progesterone secretion. The results of progesterone assays were used to construct individual secretion profiles and as reference data for evaluating the accuracy of the corresponding results obtained by rectal palpation. Both kinds of individual data were considered to be consistent with each other when no corpus luteum was detected by palpation and no progesterone secretion was present, or, when a corpus luteum was found and the corresponding progesterone concentration exceeded 2 ng/ml. The results, pooled from given intervals post partum, were expressed as relative frequency of inconsistent data, and evaluated by the so-called G-function (Sokal and Rohlf, 1969), a modified form of the chi-square test.

Results

The results of clinical findings, viz. the times of uterus involution and of detecting individual ovarian structures for the first time post partum, calving-to-conception intervals (CCI), as well as data pertaining to progesterone secretion, the beginning of secretory cycles and of possible pre-cyclic secretion, are presented in Table I. From those data correlation coefficients were calculated (Table II). The only significant, albeit not very strong, correlations were found between the length of the CCI and the time of beginning of secretory cycles ($r = 0.33$) or of progesterone secretion ($r = 0.32$).

The mean uterus involution time was 22.9 ± 4.9 days, significantly shorter in cows with an early onset of secretory cycles (ESC cows, see below; 21.9 days) than in the others (25.7 days), though the corresponding correlation coefficient was non-significant (see Table II). The remaining mean parameters are contained in Table I.

Table I

Clinical and laboratory findings in postpartum cows

Cow No.	Uterus invol. (days)	First time detected (days post partum)						No. of insem.	Calving-to-conception interval, days
		small follicles	large follicles	corpus luteum	prog. in milk	Secret. cycle	Observ. oestrus		
1	17	9	—	28	9	23	48	1	48
2	20	7	—	42	17	35	35	1	49
3	20	14	21	42	10	34	55	1	55
4	23	24	—	36	28	32	56	1	56
5	25	18	39	46	15	22	44	1	58
6	30	—	—	44	19	16	58	1	58
7	21	—	—	35	16	22	22	1	61
8	23	12	26	33	16	24	44	1	62
9	19	11	25	53	18	25	41	1	63
10	28	14	—	28	20	25	25	1	64
11	20	—	16	22	23	20	65	1	65
12	19	—	19	33	12	26	66	1	66
13	28	—	29	42	16	21	67	1	67
14	24	17	31	38	22	20	69	1	69
15	22	16	29	58	16	29	69	1	69
16	20	16	—	41	16	32	32	1	72
17	25	11	25	46	21	28	49	1	73
18	15	15	24	49	15	32	73	1	73
19	22	21	—	39	18	31	73	1	73
20	25	22	29	37	23	29	29	1	74
21	29	—	22	43	21	34	76	1	76
22	37	—	—	31	17	39	39	1	79
23	24	17	—	24	23	21	21	1	81
24	18	18	32	39	21	19	36	1	82
25	25	18	42	54	13	21	83	1	83
26	26	—	40	47	19	18	17	2	88
27	16	16	23	50	19	26	89	1	89
28	16	16	—	44	17	25	91	1	91
29	26	12	29	47	19	34	92	1	92
30	20	17	—	41	30	39	39	2	97
31	28	21	—	49	40	36	74	2	98
32	32	18	—	53	12	42	66	2	103
33	22	15	29	35	20	33	33	2	107
34	27	20	—	48	13	25	25	2	110
35	18	11	—	46	11	20	37	2	114
36	20	—	22	33	26	29	67	2	115
37	17	10	—	31	17	22	22	3	125
38	28	—	35	42	42	36	82	2	140
39	21	14	28	49	17	42	147	1	147
$\bar{x}$	22.9	15.5	27.9	40.8	19.2	28.1	55.3	1.3	81.8
$\pm s$	± 4.9	± 4.1	± 6.7	± 8.7	± 6.9	± 6.7	± 26.6	$\pm .5$	± 24.1

Progesterone secretion started between 9 and 42 days post partum, but this coincided with the first secretory cycle only in 20% of the cows studied; in the remaining 80% there was a short-lasting, pre-cyclic secretory activity. The development of ovarian structures will be presented below for the pre-cyclic and cyclic post partum periods.

Table II

Correlations among some of the parameters

	CL	PS	SC	CCI
UI	0.06	0.22	0.22	0.03
	CL	—0.10	0.22	0.19
		PS	0.26	0.32*
			SC	0.33*

Explanations: UI — uterus involution; CL — first detection of corpus luteum; PS — first detected progesterone in milk; SC — beginning of the first secretory cycle; CCI — calving-to-conception interval (all parameters expressed in days post partum): $^{*}\alpha < 0.05$

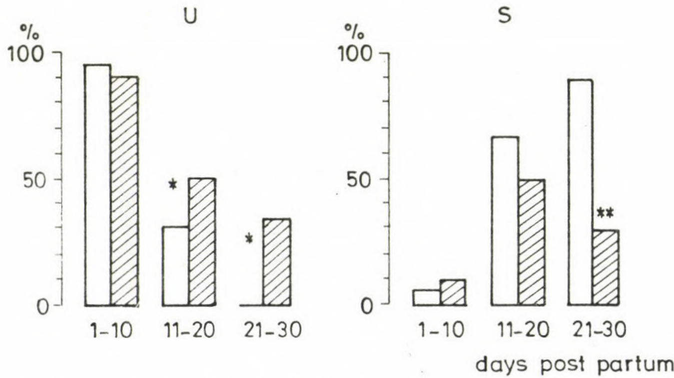

Fig. 1. Relative numbers of postpartum cows in which both ovaries were palpated as smooth (U), and of those in which multiple, small follicles were found on at least one ovary (S). Empty bars: cows with an early onset of secretory cycles (up to 32 days post partum; "ESC cows"): shaded bars: cows with a late onset of secretory cycles (more than 32 days p.p.; "LSC cows"); $^{*}\alpha < 0.05$, $^{**}\alpha < 0.01$, $^{***}\alpha < 0.001$

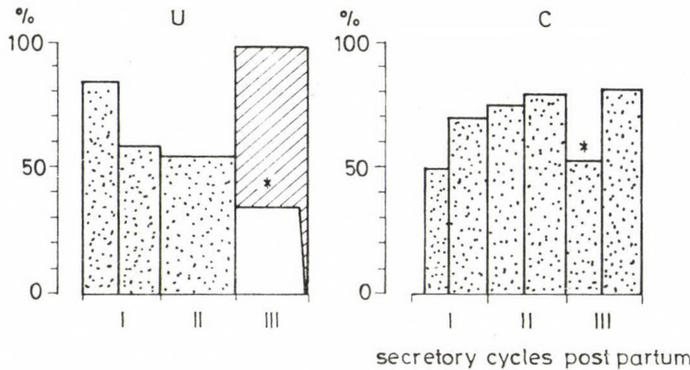

Fig. 2. Relative numbers of cows in which ovary was palpated as smooth (U), and of those in which corpora lutea were found (C). Dotted bars: all cows combined; I, II, III: consecutive secretory cycles post partum. For further explanations see Fig. 1

The pre-cyclic postpartum period (from calving to the beginning of the first secretory cycle). During the first month after delivery (but before cyclic secretory activity began) undifferentiated ovaries, or multiple small follicles were prevailing. During the first decade in nearly all animals both ovaries were smooth. Later (second and third decades), the fraction of animals with both ovaries undifferentiated decreased much faster in cows with an early (up to 32 days post partum) onset of secretory cycles ("ESC cows") than in the other ones ("LSC cows"). As shown in Fig. 1-U, in the third decade no ESC cow has both ovaries smooth, in contrast to the 33% frequency in the LSC ones. Conversely, the percentage of cows developing multiple small follicles on at least one ovary reaches in the third decade 90 in ESC animals, but only 33 in the LSC ones (Fig. 1-S). When detection of any structure, not only small follicles, was taken into account, those percentages were 100 and 67, respectively.

The period after the beginning of secretory cycles. During the first half of the first secretory cycle at least one ovary was still undifferentiated in about 85% of the cows. This decreased to 55% during the second cycle, in ESC and LSC cows alike. In the third cycle all LSC cows had one ovary palpable as undifferentiated, in contrast to 36% of ESC cows (Fig. 2-U). The frequencies of detecting small follicles were similar in all postpartum cycles, however, up to the 15th day of cycles small follicles were detected significantly less frequently in LSC than in ESC cows (42 and 23%, respectively; cf. Fig. 3-S). In the case of large ovarian follicles, the frequencies of their detection varied characteristically throughout the secretory cycle, being lowest between days 6 and 15 of cycles (20 to 31%), and highest towards the end of the cycles, but only in cows which had relatively short (up to 92 days) CCI. In other cows the highest frequency was observed in the mid-luteal phase (11–15th day, cf. Fig. 3-L).

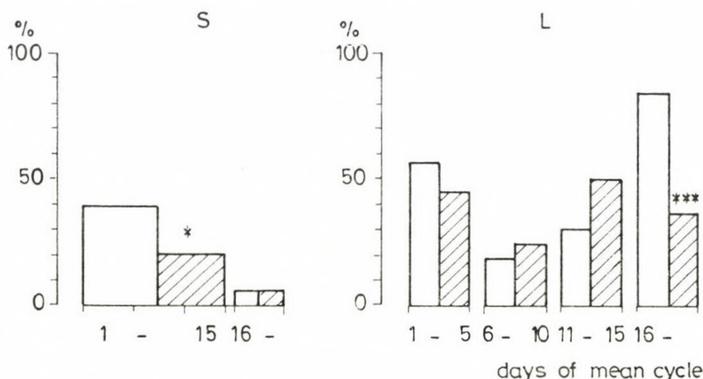

Fig. 3. Relative numbers of cows in which multiple, small follicles (S) or large ovarian follicles (L) were palpated in different phases of secretory cycles. All secretory postpartum cycles combined. Empty bars: S — "ESC cows"; L — early conceiving cows (up to 92 days post partum); shaded bars: S — "LSC cows"; L — late conceiving cows (over 92 days post partum). For further explanations see Fig. 1

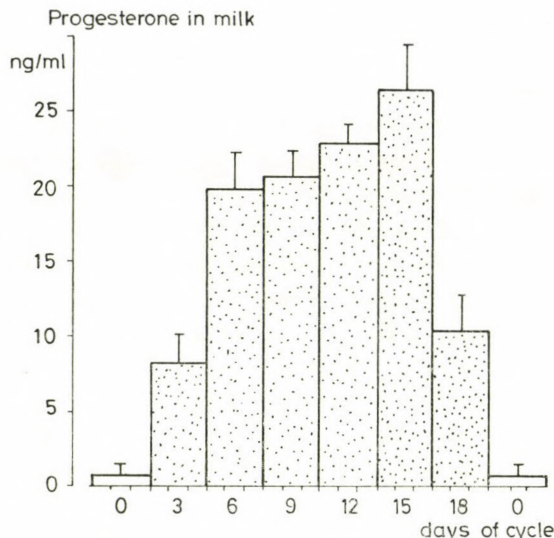

Fig. 4. Levels of progesterone in whole milk (means ± SE). All cows and cycles combined

The classification criteria of the cows had no effect on the frequency of corpus luteum detection. This increased steadily up to the end of the second secretory cycle, and then significantly decreased, increasing again towards the end of the third cycle (Fig. 2-C). The average milk progesterone profile, calculated from 51 pooled secretory cycles, is presented in Fig. 4.

Relative discrepancy between the results of rectal palpation and the progesterone secretion. The entire CCI was divided into 2 phases: early phase (from delivery up to the end of the first secretory cycle) and late phase (the remaining period, up to conception). The results were classified according to the observed ovarian structures, the presence or absence of the corpus luteum being the

Table III

A: Inconsistency of rectal palpation results with the corresponding milk progesterone levels

	Early postpartum period						Late postpartum period					
RP	N	n	%	G	n	P(ng/ml)	N	n	%	G	n	p(ng/ml)
U/U	67	8	12		8	7.1±2.8	11	10	91		10	4.9±2.0
S/U, S	71	20	28	9.11*	20	8.7±7.3	9	4	44	9.59*	4	8.0±7.1
L/U, S	44	15	34		15	8.9±6.8	36	15	42		15	7.9±6.8
C/U	40	11	28		29	11.6±9.6	29	3	12		26	11.9±9.9
C/S	25	4	16	1.55	21	11.6±9.7	22	2	9	13.04**	20	11.7±10.8
C/L	18	3	17		15	14.2±11.6	50	20	40		30	14.5±12.3

Values connected with solid lines do not differ significantly

B: Summary of discrepant results

Palpated ovarian structures		Postpartum period (N, %) Early	Late	G
U/U	(a)	67 12%	11 91%	31.5**
S, L/U, S		160 34% (b)		
C/U, S		(a) 134 17%		9.9**
C/L			50 40% (b)	

Values with the same subscript do not differ significantly
$^{*}\alpha < 0.05$ $^{**}\alpha < 0.01$ $^{***}\alpha < 0.001$

Explanations: Early p.p. period — from delivery to the end of first secretory cycle. Late p.p. period — from the beginning of second cycle to conception. RP — rectal palpation results for both ovaries: U — smooth, undifferentiated ovary, S — small follicles, L — large follicle, C — corpus luteum, e.g.: S/U, S — small follicles on one ovary, the contralateral one either smooth or with small follicles. N — total numbers of rectal examinations; n — number of discrepant observations (milk progesterone exceeding 2 ng/ml in the absence of corpus luteum, or up to 2 ng/ml with a corpus luteum present. P — progesterone concentration (mean ± S.D.), calculated only from data exceeding 2 ng/ml

principal factor. The numbers of examinations, relative discrepancies, and mean concentration of progesterone (only from values $>$ 2 ng/ml) are presented in Table IIIA. The highest discrepancy (91%) was observed in the late phase of CCI when no corpus luteum was palpable, and both ovaries were estimated as smooth. The corresponding value for the early phase was only 12%. Some groups of data which did not differ significantly were pooled and presented in Table IIIB.

Discussion

Rectal palpation constitutes the principal practical method of evaluating the state of reproductive organs in postpartum cows. In the present study attention was drawn to the morphological and functional development of ovaries. Uterus involution and other routine clinical examinations served only to follow up the course of postpartum events. It should be noted, however, that the times of first detection of ovarian structures are not exact, for rectal examinations were carried out only once weekly.*

Uterus involution and the development of ovarian structures and of progesterone secretion post partum were in agreement with the reports of others (Callahan et al., 1971; Webb et al., 1980). It is, however, interesting to note that the time of the start of ovarian secretory activity seemed to depend on the time when at least one of the ovaries was beginning to differentiate (cf. Fig. 1), although there was no direct correlation between the corresponding

* All rectal examinations were carried out by the same person (W. W.)

data contained in Table I. The increase in frequency of apparently smooth ovaries in LSC cows in the 3rd cycle (Fig. 2-U) could be due to the presence of poorly-palpable corpora lutea, since a very high proportion of discrepant results was observed at that time (90%; cf. Table II). A significant decrease in palpable corpora lutea in the first half of the 3rd cycle (Fig. 2-C), compared with the first two cycles, is consistent with that conclusion. Another interesting finding was the difference between distribution of large follicles throughout the secretory cycles in early and late conceiving cows (Fig. 3-L). The lowered incidence of large follicles at the end of the cycle in the latter ones might reflect some disturbances in the development of the pre-ovulatory follicle in those cows which might, in turn, result in the inseminations being performed at wrong times.

The analysis of palpation data as confronted with the corresponding milk progesterone values shows how variable a degree of confidence can be connected with the former ones. The observed discrepancies, as reflected by relative frequencies of inconsistent results, varied with observed ovarian structures, and with the phase of the postpartum period, ranging from 9 to 91% (Table IIIA). After pooling the numbers of non-differing observations, some clearer tendencies could be seen (Table IIIB). Highest accuracy of palpation was achieved in the early postpartum period when neither ovary contained palpable structures (only 12% of discrepant results). The same situation in the late postpartum period resulted in the lowest accuracy, however. The possible reason for this has been mentioned above. Relatively high accuracy was also attained when corpus luteum was palpable (17% of discrepant results). Only in the late postpartum period, when both corpus luteum and a large follicle were detected in the same animal, the accuracy significantly decreased (40% of discrepant results). Similarly poor accuracy (34%) was observed throughout the entire period when any ovarian structure other than corpus luteum was palpated on at least one ovary. It seems on the whole that the peri-oestrous phase of the cycle is the most difficult to evaluate by rectal palpation, and progesterone assay in milk should be carried out whenever possible to render the diagnosis more accurate.

In the literature so far reviewed few data have been found pertaining to the development of ovarian structures throughout the postpartum period. Similar results, as far as corpus luteum palpation vs. progesterone assays were concerned, were reported by Boyd and Munro (1979). Also Dawson (1975) studied the accuracy of palpating corpora lutea as confirmed upon slaughter. Elsewhere (Woyno et al., 1985) we have reported similar results obtained in slaughter cows but confronted with plasma progesterone assay results. We found a high accuracy of detecting corpus luteum, and a very low one in cases of apparently smooth ovaries, which tallies with the present findings related to the late postpartum period.

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LUTEAL AND PLASMA PROGESTERONE LEVELS IN HEIFERS AND LATE POSTPARTUM COWS*

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Relationships between the concentrations of progesterone in plasma and in corpus luteum were studied in late postpartum cows, and heifers at slaughter. The data suggest that plasma progesterone level depends on the amount of luteal tissue present, rather than on the luteal concentration of this hormone.

Keywords. Progesterone, corpus luteum, plasma, heifer, cow, post partum.

A vast literature exists pertaining to changes in plasma progesterone levels in cows during the postpartum period, as well as to different aspects of progesterone biosynthesis by the bovine corpus luteum (CL) in vitro. Little is known, however, to what extent the luteal progesterone affects the circulating level of progesterone. The aim of this work was to study the relations between various luteal parameters and plasma progesterone levels.

Materials and methods

Ovaries and blood samples were collected at a slaughterhouse from two groups of animals: 22 cows culled from herd due to prolonged postpartum period (5 to 9 months) and low milk yield, and from 27 beef heifers to serve as a kind of reference material. The ovaries and plasma samples were stored deep-frozen until dissected and analysed.

According to their appearance, corpora lutea were grouped in 4 age categories: 1 (approx. 4–8 days), 2 (9–14 days), 3 (15–19 days) and 4 (old, non-functioning corpora lutea). They were excised from the ovaries, weighed, and samples were taken for homogenization in phosphate buffer containing 0.05% albumin.

Progesterone was measured by a specific, direct radioimmunoassay (Stupnicki, 1975) in unextracted plasma or CL-homogenate. Twently μl of plasma or an equivalent of 50 μg of luteal tissue were taken per assay tube. The antiserum used (a-P/R41) was directed against 12α-hydroxyprogesterone succinate-BSA.

* This work was supported by the Project MR-II/10.1.

Results and discussion

A great variability among animals was observed regarding ovarian structure. In some instances all kinds of structures were present in one pair of ovaries, e.g. 3 corpora lutea of different ages, or several cysts accompanied by an active CL. No difference between cows and heifers could be found in this respect (cf. Table I). In the 49 CL-containing ovaries, 9 corpora lutea were located so deep that they could not possibly be detected by rectal palpation. From these, 6 were active, connected with plasma progesterone levels ranging from 1.8 to 8.9 ng/ml. On the other hand, 2 protruding corpora lutea were old, non-functioning, with accompanying plasma progesterone levels of 0.2 and 0.4 ng/ml. This may explain the observed discrepancies between plasma progesterone levels and ovarian morphology as evaluated by rectal palpation in postpartum cows (cf. Woyno et al., 1979).

Since the CL's colour is one of the factors taken into account when CL is dated, 20 corpora lutea were extracted with chloroform in order to measure colour intensity. The "colour index" (K) was expressed as optical density at 455 nm calculated for gram of luteal tissue. The values of K ranged from 0.2 to 6.5 and did not correlate with any of the studied luteal parameters ($r = 0.05$ to 0.14, see also Fig. 2).

Mean values and standard deviations of the studied parameters (CL weight, luteal progesterone concentration, total content of progesterone in CL, plasma progesterone concentration), as related to the age category of CL, are presented in Fig. 1.

The values obtained for cows do not differ significantly from those for heifers, except concentration of progesterone in 9–14 days old corpora lutea (category 2). When corrected for CL age, the mean luteal progesterone concentration was lower in cows than in heifers by about 23% ($\alpha = 0.08$). Otherwise, the parameters of the two groups of animals did not differ significantly.

Before correlation coefficients were computed, all individual data were converted to logarithms, for standard deviations were proportional to the means of logarithms. The data from non-functioning corpora lutea were omitted in order to maintain linear relationships. The simple correlation coefficients are listed in Table II, and some of the correlations are shown in Fig. 2 in the form of scattergrams.

When inspecting the correlations between plasma progesterone and luteal parameters it appears that the total content of luteal progesterone is the major factor affecting the concentration of progesterone in plasma ($r_{PT} = 0.55^{**}$ and 0.68^{**}, in cows and heifers respectively), while the effect of luteal progesterone concentration was less pronounced ($r_{PC} = 0.33$ and 0.48^{*}).

In order to gain more insight into the effects of luteal parameters on the plasma progesterone level, partial correlation coefficients were calculated.

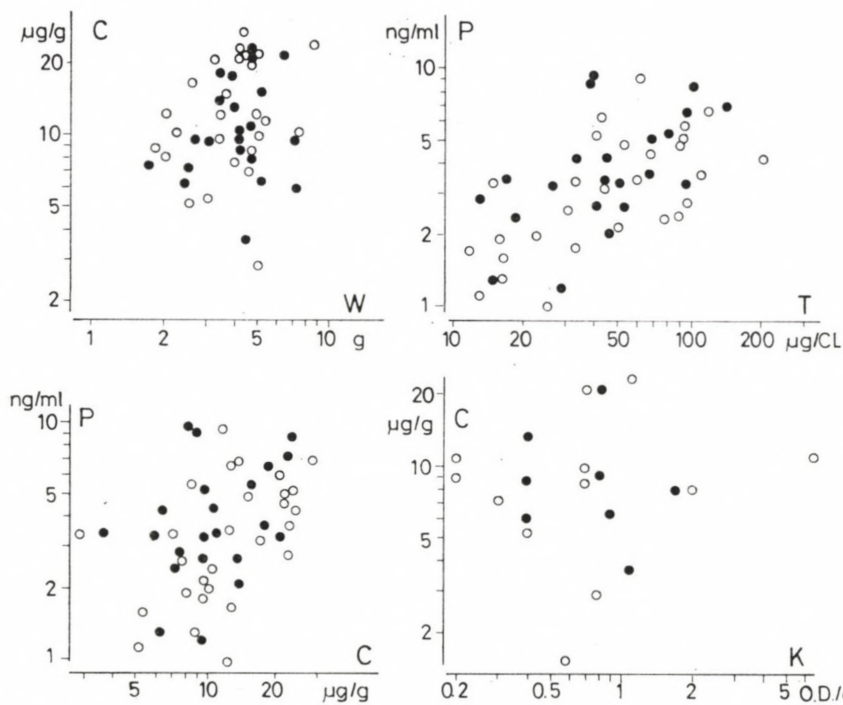

Fig. 1. Four parameters as related to the age of the corpus luteum. A = Categories of CL age. For explanation of other symbols see Fig. 2. Vertical bars denote standard deviations. Numbers of observations in parentheses

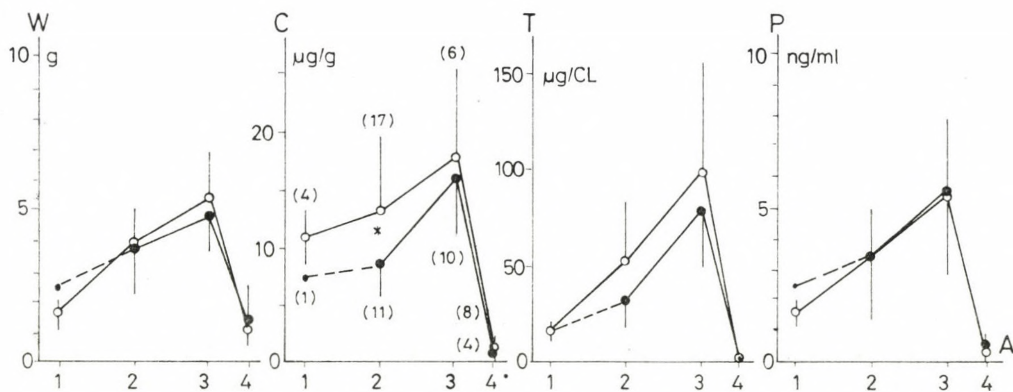

Fig. 2. Selected correlograms (log scale). ●: cows; O: heifers; W = CL weight; C = luteal concentration of progesterone; T = total luteal content of progesterone; P = plasma concentration of progesterone: K = colour index of CL

Table I

Frequency of observed ovarian structures
(means and ranges per ovary)

Ovarian structure		Cows	Heifers
Follicles:	small	3.6 (0—18)	3.1 (0—10)
	medium	5.6 (0—11)	4.6 (0—11)
	large	0.4 (0—2)	0.4 (0—2)
Follicular cysts		1.2 (0—3)	1.7 (0—6)

Table II

Simple correlation coefficients (log data)

	W	C	T	P
A	0.47* 0.75***	0.67*** 0.27	0.77*** 0.64***	0.50** 0.61***
	W	0.15 0.17	0.64** 0.72***	0.52* 0.57**
		C	0.85*** 0.80***	0.33 0.48*
			T	0.55** 0.68***

Upper line: cows; lower line: heifers; A: CL age category; W: CL weight; C: luteal concentration of progesterone; T: total luteal content of progesterone; P: plasma concentration of progesterone; * $\alpha < 0.05$; ** $\alpha < 0.01$; *** $\alpha < 0.001$

Table III

Selected partial correlation coefficients

Correlated parameters	Cows	Heifers
PW.C	0.51*	0.57**
PT.C	0.54*	0.57**
PC.W	0.30	0.47*
PC.T	—0.31	—0.15

For explanation see footnote to Table II

Some of them are presented in Table III. It can be seen that elimination of the effect of CL age or weight does not increase the correlation between plasma and luteal concentrations of progesterone in cows ($r_{CP.A} = 0.0$, $r_{CP.W} = 0.30$), while after eliminating the effect of luteal concentration of progesterone, the correlation of its plasma level with the total luteal content remains high and significant ($r_{TP.C} = 0.54$*).

The suggestion that the plasma progesterone level is affected by the luteal progesterone content more intensely than by the luteal progesterone concentration, is less conclusive in heifers, where both luteal factors significantly correlate with plasma progesterone level ($r_{PC.A} = 0.41^{*}$, $r_{PC.W} = 0.47^{*}$, $r_{PT.C} = 0.56^{**}$).

It might be speculated that the mentioned difference in luteal–plasma relations between heifers and late postpartum cows, combined with lowered luteal progesterone concentration in the latter, could be one of the factors contributing to the prolongation of the postpartum period resulting in infertility. In order to forward such a suggestion more data are needed however, especially from early conceiving postpartum cows.

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OESTRADIOL-17β INFLUENCE ON TESTOSTERONE PRODUCTION OF LUTEAL CELLS FROM EARLY PREGNANT, OESTRADIOL BENZOATE-TREATED AND HUMAN CHORIONIC GONADOTROPIN-TREATED SOWS IN VITRO

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Luteal cells separated from early pregnant, oestradiol benzoate- (EB) treated and human chorionic gonadotropin- (hCG) treated sows were incubated with or without oestradiol-17β (E_2) to estimate the influence of E_2 on testosterone (T) production in vitro. The concentrations of E_2 were 1, 10, 100, 1000 and 5000 ng/ml medium. Trypsin-dispersed luteal cells, 5×10^4 cells/ml, were suspended in Eagle's medium enriched with 2% human serum albumin. The T amounts in the medium samples were estimated by a radioimmunological method after an incubation of 1, 3 and 6 h.

The luteal cells from the three groups of sows released similar amounts of T during incubation: 82.5 ± 10.9, 61.4 ± 7.1 and 85.2 ± 13.0 pg/ml, respectively. The cells from pregnant pigs decreased T secretion in the presence of 10 ng E_2 per ml medium in the first hour of incubation and increased it in the presence of the highest E_2 concentration in the third hour of the experiment ($P < 0.05$). E_2 did not change the T production of luteal cells obtained from the remaining two groups (EB- and hCG-treated sows, respectively).

Keywords. Oestradiol-17β, luteal cells, testosterone, sow, pregnancy, pseudopregnancy.

Recognition of pregnancy in the sow is based on the fact that oestrogen secreted by blastocysts appears in the maternal peripheral plasma on the 16th or 17th day after mating (Robertson and King, 1974). Oestrogen as well as hCG injections prolong the life span of the corpus luteum (Garbers and First, 1969; Guthrie and Rexroad, 1981) by preventing luteolysis (Bazer and First, 1983). Moreover, exogenous hCG is followed by a rise in plasma E_2 level (Guthrie and Bolt, 1983), therefore one can suppose that this steroid is mediated by the hCG action on the corpus luteum.

During pseudopregnancy, E_2 is produced only by the corpus luteum, whereas in pregnancy also by blastocysts (Gadsby et al., 1980). High E_2 level in peripheral plasma, both in pregnancy (Robertson and King, 1974; Robertson et al., 1978) and pseudopregnancy (Johmen et al., 1980; Guthrie and Rexroad, 1981; Guthrie and Bolt, 1983), may influence steroidogenesis in the corpus luteum.

In in vitro studies E_2, considered to be a luteostatic factor (Bazer et al., 1982), did not affect the progesterone production of luteal cells obtained from

early pregnant sows (Watson and Walker, 1978), but increased it in those obtained from cyclic pigs (Gregoraszczuk, 1983).

It is known that ovarian steroids including E_2, which is formed from T by aromatization (Grower and Cooke, 1983), can influence steroidogenesis by local action. Taken this into consideration it seems reasonable to investigate if E_2 can influence T production. So far no data have been available on this subject in the pig.

The aim of this study was to examine E_2 influence on the T production of luteal cells obtained from early pregnant, EB-treated and hCG-treated sows in vitro on the 18th day of pregnancy or pseudopregnancy.

Materials and methods

Animals. The sows were obtained from a commercial pig fattening farm. Day zero of the oestrous cycle was established on the basis of the tolerance reflex. The sows were assigned randomly to three groups as follows: group 1: non-injected pregnant sows (n = 4); group 2: sows treated with oestradiol benzoate (Polfa, Poland), 5 μg/day, between days 11 and 15 of the oestrous cycle (n = 5); group 3: sows treated with 1000 IU hCG (Biogonadyl, Laboratories of Sera and Vaccines, Poland) on day 12 of the oestrous cycle (n = 4). The sows were killed on the 18th day of pregnancy or pseudopregnancy. After dissection, the ovaries were immediately transferred into cold (4 °C) sterile PBS (Laboratories of Sera and Vaccines, Poland) containing 50 U penicillin and 50 μg streptomycin per ml.

Separation. Luteal cells were separated by a technique recommended by Stouffer et al. (1976), i.e. dispersion in 0.25% trypsin solution (Laboratories of Sera and Vaccines, Poland). Cells were counted in a haemocytometer, using trypan blue stain (1%). The viable cells were suspended up to 5×10^4/ml in Eagle's medium (Laboratories of Sera and Vaccines, Poland) enriched with 2% human serum albumin (fraction V).

Incubation. Each cell suspension was distributed in Leighton tubes which were then incubated at 37 °C for 1, 3 and 6 h, in duplicates. In addition, three samples in each experiment were frozen without incubation to estimate the endogenous (pretreatment) T content. The luteal cells were incubated without E_2 or with E_2, 1, 10, 100, 1000 or 5000 ng/ml (Sigma, USA).

Radioimmunoassay. The medium samples were stored at —20 °C until assayed. T concentration was estimated by the method described by Thompson et al. (1977), except that 0.5-ml samples were extracted with 4 ml diethyl ether. Tracer T [(1, 2, 6, 7) -^{3}H-testosterone, Radiochemical Centre, Amersham, U. K., spec. act. 107 Ci/mmol], and "cold" testosterone (Sigma) were used. Highly specific antibodies against testosterone were kindly provided by

Prof. R. Rembiesa (Institute of Pharmacology, Polish Academy of Sciences, Cracow). T antibodies cross-reacted with 5α-dihydrotestosterone (50%); 5α-androstan-3α, 17β-diol (28%); 5α-androstan-3β, 17β-diol (4.28%); androstendione (2.83%); 5α-androstan-3, 17 dion (2.6%); 5-androsten-3β, 17β-diol (1.03%); androsterone (0.85%); epitestosterone (0.42%); epiandrosterone (0.16%); 5β-androstan-3,17 dion (0.10%); dehydroepiandrosterone (0.06%); oestradiol-17β, pregnenolone, progesterone, corticosterone, hydroxycortisone (0.01%). T recovery rate was estimated as 92.4%. The sensitivity of the assay was 5 pg per tube. The coefficients of variation within and between assays were 4.98% and 10.21%, respectively.

Statistics. A split-plot analysis of variance (Gill and Hafs, 1971) was used to compare testosterone concentration in control cultures (without E_2) and E_2-treated cultures in the 1st, 3rd and 6th h of incubation as well as to test differences in testosterone level during incubation. E_2 doses were used as the first variable and time of incubation as the second one. Comparison of means between the doses studied was performed by Duncan's multiple range test.

Results

The endogenous (pretreatment) T concentration in the samples before incubation of luteal cells from sow groups 1, 2 and 3 was 60.8 ± 18.4, 44.8 ± ± 7.6 and 60.0 ± 12.4 pg/ml, respectively. The average T level in samples incubated for 1, 3 or 6 h without E_2 amounted to 82.5 ± 10.9 for group 1, 61.4 ± 7.1 for group 2 and 85. 2 ± 13.0 pg/ml for group 3.

Table I presents the T amounts produced by the luteal cells obtained from group 1 of sows. E_2, 10 ng/ml, inhibited ($P < 0.05$) T production in the

Table I

Influence of various oestradiol-17β (E_2) concentrations on testosterone production (pg/ml) of luteal cells obtained from early pregnant sows*

E_2 concentration (ng/ml)	Incubation time (h)			Mean T amount for E_2 concentrations, pg/ml
	1	3	6	
0	105.9±26.3[a]	69.7±12.7[a]	71.9±13.7[ab]	82.5±10.9[ab]
1	65.1±19.0[ab]	51.2±17.3[a]	68.0±20.6[ab]	61.5±10.2[a]
10	59.1±17.5[b]	64.5±12.7[a]	64.5±7.3[a]	62.7±6.9[a]
100	72.4±14.7[ab]	90.7±25.2[ab]	108.7±37.2[ab]	90.6±15.0[ab]
1000	116.8±24.8[a]	81.1±9.8[ab]	119.8±28.1[b]	105.0±12.8[ab]
5000	96.6±30.8[ab]	167.3±14.2[b]	137.8±28.4[b]	134.6±15.8[b]
Mean T amount for incubation time (pg/ml)	86.4±9.4	87.4±9.8	95.2±10.7	—

* The values are the means of 4 experiments ± SEM. Values in the same line followed by different letters are significantly different ($P < 0.05$)

Table II

Influence of various oestradiol-17β concentrations on testosterone production (pg/ml) of luteal cells obtained from sows treated with oestradiol benzoate at a dose of 5 μg/day between days 11–15 of the oestrous cycle*

E_2 concentration (ng/ml)	Incubation time (h) 1	3	6	Mean T amount for E_2 concentrations (pg/ml)
0	58.1±14.1	69.9±12.8	56.1±11.9	61.4±7.1
1	59.8±18.8	51.1±18.2	50.4±10.4	53.8±8.8
10	47.7±7.5	55.4±11.1	54.4±14.5	53.5±6.2
100	57.6±17.1	69.9±10.5	62.6±13.1	63.4±7.5
1000	57.8±14.0	53.5±9.5	62.5±19.5	57.9±8.0
5000	50.7±13.7	45.9±14.2	76.5±23.3	57.7±10.1
Mean T amount for incubation time (pg/ml)	55.3±5.5	57.6±5.1	60.9±6.2	—

* Values are the means of 5 experiments ± SEM

Table III

Influence of various oestradiol-17β concentrations on testosterone production (pg/ml) of luteal cells obtained from sows treated with 1000 IU of human chorionic gonadotropin on day 12 of the oestrous cycle*

E_2 concentration (ng/ml)	Incubation time (h) 1	3	6	Mean T amount for E_2 concentrations (pg/ml)
0	74.5±20.6	95.8±27.1	85.4±24.2	85.2±13.0
1	62.5±14.1	66.7±21.5	81.0±28.0	70.1±11.7
10	55.1±15.6	82.8±27.0	129.9±48.2	89.3±19.6
100	84.0±21.4	89.1±25.1	71.9±19.9	81.7±11.8
1000	63.6±17.0	101.2±16.8	110.2±24.9	91.7±12.0
5000	127.5±19.4	70.0±8.2	97.3±5.4	98.3±9.7
Mean T amount for incubation time (pg/ml)	77.9±8.3	84.3±8.4	96.0±10.9	—

* Values are the means of 4 experiments ± SEM

first hour of incubation, whereas 5000 ng/ml stimulated ($P < 0.05$) it by the end of the third hour. The significant differences ($P < 0.05$) in T concentrations occurred between samples where E_2 concentrations of 1 and 10 ng/ml and extremely high concentrations (5000 ng/ml) were used.

Luteal cells from groups 2 and 3 did not change T production under E_2 influence (Tables II and III).

Discussion

The endogenous T concentrations as well as T amounts in the samples during incubation without E_2 were similar in the three physiological stages under study. However, the luteal cell reactions on E_2 action were different.

The luteal cells from pregnant sows decreased T production in the presence of the lowest E_2 concentrations early in the incubation and increased it in the presence of the highest E_2 concentration subsequently. The results suggest that low E_2 doses increase T conversion, whereas the highest dose causes T accumulation in luteal cells. None of the E_2 doses used affected the T production of the luteal cells obtained from EB-treated and hCG-treated sows.

Recent studies by Grażul et al. (1986) have shown that E_2 changed progesterone secretion by luteal cells obtained from pregnant sows but did not change it if the cells were derived from EB-treated or hCG-treated ones. In the pregnant sows E_2 plasma level (Moeljono et al., 1977) is lower than in oestradiol valerate-treated (Frank et al., 1977) or hCG-treated (Guthrie and Bolt, 1983) ones. One may suppose that high E_2 level in vivo limits E_2 influence on steroidogenesis in luteal cells in vitro.

It is known that T is released by ovarian cells during pregnancy and that LH regulates its secretion in the rat (Sridaran et al., 1981). However, the role of T during pregnancy is not clear; it seems that it is utilized by cells mainly as a source of E_2. The corpus luteum of the rat, unlike that of the cow (Henderson and Moon, 1979) has a high aromatase activity in pregnancy (Elbaum and Keyes, 1976). The porcine corpus luteum produces E_2 during both the oestrous cycle and pregnancy (Lemon and Loir, 1977; Watson and Patek, 1979), suggesting an aromatase activity. Gibori and Keyes (1978) supposed that T itself might exert direct actions in rat luteal cells, and Keyes et al. (1980) showed luteotropic effect of T in the pregnant rat.

Presumably, E_2 can influence the T secretion by ovarian cells, though considerable discrepancies occur between experimental data. Magoffin and Erickson (1981) showed that exogenous E_2 acts directly on rat ovary to abolish the hCG stimulation of androgen production by rapidly inhibiting 17α-hydroxylation. Moreover, oestrogen in high concentration inhibits LH-stimulated follicular androgen production in the rat in vivo (Leung and Armstrong, 1980) and in porcine thecal cells in vitro (Tsang et al., 1979). On the other hand, in luteal cells cultured from pregnant rats exogenous E_2 did not affect T and progesterone production (Wada et al., 1984).

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STUDY OF PROLONGED VIRUS INFECTION IN CATTLE STOCKS INFECTED BY BOVINE RESPIRATORY SYNCYTIAL VIRUS

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An outbreak of a respiratory disease caused by bovine respiratory syncytial virus (BRSV) and appearing as chronic pneumonia accompanied by complications was observed in the calf-rearing unit of a large-scale cattle farm. Among 6 to 18 months old fattening bulls of the same farm an acute respiratory disease caused by the same virus occurred. The aetiology was elucidated by virus isolation and testing paired sera. It is suggested that the severe, acute pulmonary emphysema of the 6 to 18 months old animals was due to a hypersensitivity response of allergic (immunopathological) background, since the affected animals had possessed neutralizing antibodies to BRSV already 3 weeks before they fell ill. Among the fattening bulls the outbreak passed off in 3 weeks' time; on the contrary, among the calves improvement was observed only 4 months later, with the beginning of warm weather. It is postulated that BRS virus kept circulating in the calf population even after the disappearance of clinical symptoms, since most of the susceptible calves introduced into the stock developed antibodies to BRSV without showing clinical symptoms. Contrarily, the susceptible animals introduced into the fattening bull stock failed to seroconvert, a fact that speaks against the circulation of the virus in the stock.

Keywords. Bovine, respiratory syncytial virus, infection, calf, fattening bull, pneumonia, pulmonary emphysema.

Bovine respiratory syncytial virus (BRSV) infection of cattle and the consequent respiratory disease have been described all over the world (Paccaud and Jaquier, 1970; Wellemans et al., 1970; Inaba et al., 1970). The incidence, course, clinical symptoms and pathological lesions of the disease as well as the possibilities of its diagnosis and control have been reported (Rossi and Kiesel, 1974; van Nieuwstadt and Verhoeff, 1983). The occurrence of BRSV in Hungary and its role in the aetiology of the so-called epizootic coughing (yearling pneumonia) of cattle were reported in 1975 (Köves and Bartha, 1975); the same virus has been incriminated, in addition to the above-mentioned syndrome, in the aetiology of the complicated pneumonia of young calves showing no characteristic features. Serological surveys have revealed that a considerable part of large-scale cattle stocks in Hungary are infected by BRSV. In most of these stocks, in certain age groups, infections pass off without any symptom. As asymptomatic infection of certain groups of cattle was observed over periods in several large-scale cattle stocks, the supposition was close at hand that permanent infection was established by animals that,

for some reason or other, had become virus excretors. The factors to which virus circulation in a given stock may be attributed are, however, unknown; neither do we know where and how the virus persists in the stock. In the present paper we report on our studies concerning the circulation of BRSV infection in different age groups of a large-scale cattle stock after an outbreak of BRSV-induced respiratory disease, and on the time-course of the infection.

Materials and methods

Description of the disease

In the specialized cattle unit of farm "D" 200 Limousine x Hungarian Fleckvieh F_1 cows and their progeny are kept. Heifer calves are reared for replacement of the cow stock, while bullcalves are fattened on an intensive feeding regime, under loose housing conditions, grouped by age, 20 bulls/group, from 6 to 18 months of age. Usually 10 days old, occasionally also older, bullcalves are purchased from both smallholders and large-scale farms for fattening. These are introduced into the appropriate age group of the farm's own stock after an isolation period which usually lasts 30 days.

As in the calf-rearing unit bovine viral diarrhoea (BVD), infectious bovine rhinotracheitis (IBR) and adenovirus infection, infections causing considerable economic losses, had become permanent, vaccination against these three diseases was started 10 years ago and has been practised continuously ever since according to the regulations; the purchased animals are also vaccinated. As a result of vaccination, the formerly 10 to 20% calf losses have been reduced to 1 to 5%, which ensures an economical operation.

In November 1982, a respiratory disease emerged in the calf-rearing unit, among the 3 to 4 months old calves. As regards its course, clinical symptoms and pathological lesions, it resembled the disease observed in the stock in the early 'seventies, a disease attributed to IBRV and adenovirus infection. The disease was practically eliminated later by regular vaccination. The infection spread slowly among the calves; after two months all the animals, except those younger than 6 weeks and introduced after the outbreak, showed clinical symptoms indicative of respiratory disease. The disease occurred in both the farm's own calves and the purchased ones. Clinical symptoms persisted over a long period, frequently even for 4 to 6 weeks. The development of clinical symptoms was best observable on the 40 to 50 days old purchased calves introduced into the affected calf house. On days 5–10 after introduction, these calves showed tachypnoea, serous nasal discharge and lacrimation. The affected calves had a temperature around 40 °C, and within a few days they developed an aggravating dyspnoea, had a frequent and wet cough, and first a mucous, then a purulent, nasal discharge. Their haircoat was mat and shaggy.

From the third week onwards, their body condition rapidly deteriorated and their growth was retarded. In this case none of the calves died in the acute stage of the disease; deaths occurred usually in the 4th or 5th week, as a result of secondary pleuropneumonia.

In the calf house the disease lasted 4 months; affected calves started to decrease in number only when the introduction of calves born in the meantime was stopped. In the winter period, 14 of the 180 calves (7.7%) died. Cows and heifers housed in separate barns of the specialized farm showed clinical symptoms neither at the time when the calves became affected nor later on. Subsequently, in the warm season, lasting from April to September, the disease practically disappeared, although a few calves exhibited mild respiratory symptoms which, however, soon subsided. With the onset of the cold season, in November, disease cases occurred again, but in a much milder form. In that winter 9 calves (4.5%) died of a respiratory disease accompanied by symptoms similar to those observed in the previous year.

In March 1983, when much fewer affected calves were kept in the calf-rearing house, a sudden outbreak of acute respiratory disease occurred in a fattening bull barn, 500 m away from the calf house, among 7 months old fattening bulls transferred here from the infected calf house 6 weeks earlier. The disease spread over to the whole fattening bull stock within a week; nearly all the bulls showed clinical symptoms characteristic of the respiratory disease known as "yearling pneumonia" or "epizootic coughing". The animals coughed very frequently and loudly, their body temperature rose as high as 41 °C, they showed abundant serous nasal discharge and dyspnoea. Some animals exhibited emphysema in the subcutaneous connective tissue of the dorsal region. Most of the affected bulls recovered within 3 to 5 days, but four 8–10 months old animals (2%) died. Twenty days after the occurrence of the first case the whole stock was healthy again, and no relapses occurred even subsequently. Thirty days after the disease had disappeared, the introduction of bullcalves that had reached the age of 6 months in the calf house or had been purchased from smallholders, was begun and continued later on as well. No respiratory or any other disease was observed among the introduced fattening bulls during the two-year period of observation.

Serological examinations

The first blood samples taken in the calf house were received 8 weeks after the disease had emerged, in mid-January, 1983. Blood samples were taken from 5 months old calves that had been affected earlier but recovered since, and from freshly affected 8 weeks old calves purchased from smallholders. Second blood samples of the same animals were sent to the laboratory 3 weeks later, together with those taken from the — at that time still healthy

— bullcalves. Subsequently, blood samples were taken and tested regularly from animals that had recovered and from BRSV-antibody-free animals purchased from smallholders and introduced into the calf and fattening bull stock at 10 days and 6 months of age, respectively. BRSV neutralizing antibodies were determined in an FLK cell line carrying bovine leucosis virus. Haralambiev's method (Haralambiev et al., 1982) was used.

Antibodies to parainfluenza-3 (PI-3) virus were examined by the haemagglutination-inhibition (HI) test in the usual way. Since the cattle stock was continuously immunized with IBR, BVD and adenovirus vaccines the sera were not tested for antibodies to these viruses.

Gross and histopathological examination

From calves of smaller body size the entire carcase, while from the larger fattening bulls only the pathologically-altered organs were submitted for examination. In submitting test material to the laboratory, preference was given to carcases of animals that had died of acute symptoms after a rapid course. The histopathological sections prepared from the pathologically-altered parts were stained with haematoxylin and eosin. Occasionally, Braun–Brenn's staining, Giemsa staining and PAS reaction were also used.

Virus isolation

Virus isolation was attempted in secondary calf kidney epithelial cell cultures from the pathological secretions of acutely diseased calves and fattening bulls and from the pathologically-altered organs of animals that had died after a rapid course; the procedure reported earlier was used (Köves and Bartha, 1975).

Results

In January 1983, we succeeded in isolating in secondary calf kidney epithelial cell cultures a respiratory syncytial virus from nasal swabs of a calf, one of the purchased calves introduced into the infected calf-rearing house at 6 weeks of age. These calves developed acute respiratory symptoms including serous nasal discharge after having stayed in the calf-rearing house for 5 to 10 days. The virus-infected cell cultures contained syncytia and in the stained preparations cytoplasmic inclusions were seen. The virus-containing fluid failed to agglutinate guinea-pig red blood cells. We identified the virus by the virus-neutralization test, using anti-BRSV serum. Further virus isolation attempts made from diseased animals of the calf-rearing house and fattening bull barn failed.

In the succumbed calves, catarrhal-purulent pneumonia and subacute pleuritis caused by corynebacterial and streptococcal superinfections, were found. The bronchial epithelium was covered by a debris-containing, purulent-mucous exudate. In some calves widening of the pulmonary septa and mild interstitial emphysema were found. In the lungs of some calves, at the boundaries of intact and pathologically-altered areas there were multinuclear giant cells (syncytia) in the alveolar and bronchial epithelial cells. Neither intranuclear nor cytoplasmic inclusions were found in the lungs of calves died of the respiratory disease.

The lungs of fattening bulls died of respiratory symptoms after a short disease course (2 to 3 days) did not collapse after exposed; thus, it seemed larger than its normal size. In the lungs there was a severe interstitial emphysema; the alveoli reached the size of a fist or a child's head. The pulmonary septa were strikingly widened and infiltrated with serous exudate and contained gas bubbles. The tracheal mucosa was scarlet red. Histopathologically, there was a serous exudation in the interlobular connective tissue; other findings included interstitial pneumonia, acute bronchitis, fusion of certain bronchial and alveolar epithelial cells (syncytium formation), and cytoplasmic inclusions in some respiratory epithelial cells.

The titres of BRSV neutralizing antibodies in sera of diseased calves and fattening bulls are shown in Table I. Table II gives data on the occurrence and titres of antibodies in purchased susceptible calves introduced into the calf house after the outbreak had passed off. Table III shows that in growing animals introduced into the fattening bull barn, where recovered animals were kept, virus-neutralizing antibodies failed to appear during the long period of observation. Antibodies to PI-3 virus were demonstrable by the HI test in about a half of the sera tested. Since the occurrence and titres of HI antibodies were as usual, they are not indicated separately.

Table I

BRSV antibodies in calves and fattening bulls

Age of the animals at the first testing	Onset and end of the disease outbreak	Dates of blood sampling		
		15 January	5 February	10 April
5 months old "own" calves	15 November– –5 January	10/10* 1 : 56+	10/10 1 : 48	
8 weeks old purchased calves	12 January– 20 February	0/10	10/10 1 : 42	8/10 1 : 23
6 months old "own" fattening bulls	1 March–20 March		7/10 1 : 32	10/10 1 : 92

Numerator: number of positive animals; * denominator: number of animals tested; + mean antibody titres in the positive sera

Table II

BRSV antibodies in purchased calves introduced into the calf-rearing house after the outbreak had passed off

Date of introduction	Dates of blood sampling			
	15 April	1 July	1 October	1 December
15 April	0/9	6/9 1 : 22	8/9 1 : 19	
1 July		0/10	7/10 1 : 42	8/10 1 : 28
1 October			0/7	7/7 1 : 46

For explanations, see Table I

Table III

BRSV antibodies in purchased growing bulls introduced into the fattening bull barn after the outbreak had passed off

Date of introduction	Dates of blood sampling			
	4 May	1 September	15 January	15 March
4 May	0/10	0/9	0/7	0/10
1 September		0/5	0/5	0/4
15 January			0/8	0/6

For explanation, see Table I

Discussion

The present studies have shown that the respiratory disease in the calf-rearing house and fattening bull barn of farm "D" was due to BRSV infection. This is supported by successful virus isolation, the results of testing paired sera and the pathological lesions.

Isolation of the virus is not considered a precondition of diagnosing the respiratory disease caused by BRSV, because BRSV is very difficult to isolate (Lehmkuhl et al., 1978). We succeeded in isolating the virus only in 20% of cattle stocks affected with respiratory disease undoubtedly caused by BRSV. The isolation rate of BRSV is even worse if the number of virus isolations is related to the number of samples tested; only 1% of the samples yielded virus. Also in farm "D" only one sample yielded BRSV although isolation was attempted from numerous samples over a long period. It is remarkable that in the farm the virus was isolated from nasal secretions of a freshly-diseased susceptible calf that had been purchased from smallholders and introduced

into the infected calf-rearing house 10 days before the sample was taken. Virus isolation from the farm's "own" animals diseased either in the calf-rearing house or the fattening bull barn failed consistently.

According to literary data, it is advisable to base the diagnosis of BRSV-induced respiratory disease on testing the antibody titres of paired sera. According to our experiences, this recommendation can be accepted only with certain reservations, since in a considerable part of our cases we failed to demonstrate a significant antibody titre increase, for the first serum sample of diseased animals contained antibodies; these were of maternal origin or resulted from an earlier asymptomatic infection (Holzhauer, 1978). It has been suggested that the blood withdrawn from diseased animals on the 3rd day of the disease might already contain maximum antibody titres (Baker and Frey, 1985); thus, it would be unjustified to base the diagnosis on titre rise in paired sera. In farm "D" the blood testing started in mid-January had no diagnostic value in calves that had fallen ill two months earlier, since by that time all the calves tested possessed antibodies. Testing paired sera may have diagnostic value only when the first sample is taken on the very first days of the disease, since on the subsequent days seronegative animals may develop antibodies. However, in the present study the paired sera taken from the purchased, susceptible calves introduced into the infected calf-rearing house 10 days earlier did have diagnostic value (Table I).

The serological examinations made in the fattening bull stock of the farm were sufficient to assume the aetiological role of BRSV. Table I shows that more than a half of the blood samples taken 3 weeks before the outbreak contained antibodies in a mean titre of 1 : 32. These antibodies could not have been of colostral origin but must have appeared as a result of an earlier asymptomatic infection. All the postepidemic blood samples contained antibodies which showed a nearly threefold, i. e. significant, increase in titre.

The gross pathological lesions seen in calf carcases were not characteristic; they did not differ at all from lesions occurring as a result of IBRV or bovine adenovirus infection. Specific histopathological changes were infrequent and, thus, unsuitable for routine diagnostic purpose.

On the other hand, in the fattening bulls died of the disease there were gross and histopathological lesions typical of BRSV-induced respiratory disease. The marked widening and oedematous infiltration of interlobular septa, the appearance of numerous large air bubbles in the emphysematous lungs and, histopathologically, the fusion of a part of the bronchial and alveolar epithelial cells, the formation of multinuclear giant cells and the presence of cytoplasmic inclusions in part of the cells are findings which cannot be found together in respiratory diseases caused by either IBRV or bovine adenoviruses.

In agreement with our earlier experiences, there were striking differences between the respiratory disease of calves and fattening bulls of farm "D" as

regards course, clinical symptoms and pathological lesions. Among the fattening bulls the disease occurred in the form of the so-called "epizootic coughing" or "yearling pneumonia", whereas in the calves the pathological picture was dominated by a characterless, chronic, secondary pneumonia, a finding usual after IBRV and adenoviral infections as well. Baker and Frey (1985) regarded this clinical picture as the first stage of the disease emerging after BRSV infection, i. e. as that resembling the so-called shipping fever. On the other hand, the disease entity named by us "epizootic coughing" or "yearling pneumonia" resembles the hypersensitivity response considered by Baker and Frey (1985) to represent the second stage of the disease. Based upon the clinical symptoms and gross and histopathological lesions, also we incriminated certain allergic (immunopathological) processes in the pathogenesis of the acute respiratory disease occurring in fattening bull stocks, i. e. the so-called "epizootic coughing" or "yearling pneumonia". Our investigations into this subject will be reported in a separate paper; here we merely refer to the fact that the sera of fattening bulls contained VN antibodies already 3 weeks before the outbreak (Table I). If the sudden and rapid outbreak occurring among fattening bulls really had an allergic background and was due to a hypersensitivity response, at the time of infection a certain part of the animals must have possessed not only antibodies but also immunological memory cells converted by BRSV.

The disease that attacked fattening bulls differed from that observed in the calf-rearing house not only in its character but also in the serological response of susceptible animals introduced into the stock after the outbreak had passed off. While no VN antibodies to BRSV appeared during the nearly one-year period of observation in the sera of susceptible animals continuously introduced into the fattening bull barn (Table III), most of the calves introduced into the calf-rearing house seroconverted (Table II).

From the facts outlined above we have drawn the conclusion that after the acute respiratory outbreak of short duration and accompanied by characteristic symptoms had passed off in the fattening bull barn, persistent (prolonged) virus infection did not develop in the stock. In contrast, from the results of the serological tests it is probable that after the respiratory disease appearing among calves in the form of a prolonged, chronic, complicated pneumonia had subsided, infective virus remained present and circulated in the stock.

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BOOK REVIEW

ASHDON, R. R., DONE, S. and BARETT, S. W.: *Topographische Anatomie der Wiederkäuer.* Ferdinand Enke Verlag, Stuttgart, 1984. 238 pages, 647 colour photographs, large atlas format, in stiff plastic biards. Price: 147 DM.

The atlas is the first volume (The Ruminants) of a series *(Colour Atlas of Veterinary Anatomy)*, translated into German by Professors Margit and Bertram Schnorr.

It follows the systematization used by topographic anatomy. The first photographs of the various chapters depict the visible and palpable organs and parts of organs, which are marked as areas with the hair cut off. Palpable bone surfaces of the skeleton are indicated by painting them red. Although in this case such a representation is justified by the necessity of uniformity, more realistic pictures can be taken of live animals, since palpational anatomy is taught on live animals, too.

The stratigraphic topographic-anatomical sequences of photographs were taken of carcases fixed in standing position, in which the topography and colour of the live animals' organs was maintained in a realistic manner. The arteries were injected with neopren latex plastics. The authors deserve praise for their preparations, the relatively well-preserved colour of the organs and, first of all, for the lifelike tone of the colour photographs.

The drawings complementing, i.e. made of, the colour photographs facilitate an easy and rapid perspicuity. The vivid and intense colour of the essential formations (arteries, veins, nerves, lymph nodes and glands) makes the atlas easy to use. The photos presenting the topography of thoracic and abdominal organs speak of a nice and painstaking preparator's work. It is a pity that the authors failed to remove muscle and/or connective tissue from minor portions of a few nerves and blood vessels (4.17, 4.29), since if they had done, both the photos and the preparations would have been perfect. At the same time, the regions depicted on some close-up shots indicate the authors' extraordinary expertness in the work of preparation. Summing up the above-mentioned facts, this is the most beautiful topographic-anatomical atlas published so far.

The atlas elaborates the topographic anatomy of ruminants in its entirety in a scholarly manner, and can be used by students of veterinary science, veterinary practitioners, clinicians, surgeons and, owing to the nomenclature applied, also by those unfamiliar with the German language.

COMING EVENTS

Bicentenary of the Budapest University of Veterinary Science

The Budapest University of Veterinary Science will celebrate the 200th anniversary of the beginning of veterinary education in Hungary on *May 25–29, 1987.*

Further to the usual festivities, round table conferences and poster sessions, as well as exhibitions of instruments and drugs will be held during the bicentenary period, depending on announcements of participation, in the following topics: *physiology, morphology, pathology, epizootiology-microbiology, parasitology, animal hygiene, animal husbandry and nutrition, pharmacology-toxicology, clinical veterinary medicine, health problems of wildlife and Zoo animals.* Kindly announce your participation in the celebrations, and/or the title of your short (10 min) research paper or poster to the President of the Organizing Committee until January 31, 1987, at the latest.

The registration fee, US $ 120.—, will cover the expenses of the bicentenary publications, receptions, excursion, etc.

Address of the President of the Organizing Committee: Prof. Dr. G. Pethes, Budapest VII. Landler J. u. 2; mailing address: P. O. Box 2, H-1400 Budapest. Telephone: 223-044, or 222-660, extension 131. Telex: 224439.

German Veterinary Medical Society, Inc.

The 17th Congress of the German Veterinary Medical Society will be held from 1 to 4 April 1987 in Bad Nauheim, Federal Republic of Germany.

Leading Theme 1: Prophylaxis and Therapy in Livestock
Leading Theme 2: Topics of Veterinary Medical Research

Submissions for short communications (max. 10 minutes) to Leading Theme No. 2 and Poster Presentations of both Leading Themes are invited to the President of the German Veterinary Medical Society, Prof. Dr. Dr. h. c. mult. Anton Mayr, Institut für Mikrobiologie und Seuchenmedizin, Veterinärstrasse 13, D-8000 München 22. The deadline is 30 September 1986.

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Acknowledgement of grants and technical help.

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- for journals: names and initials of all authors, year of publication (in parentheses), colon, English title of the paper (if the original title is not English, indicate in parentheses, e.g. [in French]), journal title abbreviated according to the style used in Index Veterinarius, volume number, issue number in parentheses, first and last pages;
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