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CROSS-REACTIONS BETWEEN *MYCOBACTERIUM AVIUM* AND *MYCOBACTERIUM PARATUBERCULOSIS* STRAINS IN COMPLEMENT FIXATION AND GEL PRECIPITATION TESTS

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(Received November 19, 1983)

Sera of 45 rabbits immunized with *Mycobacterium avium* reference strains belonging to 19 serotypes were tested with antigens prepared from a *Mycobacterium paratuberculosis* strain in the complement fixation and gel precipitation tests.

Rabbit sera produced against almost all *M. avium* strains gave positive reaction with the *M. paratuberculosis* antigen in the complement fixation test. In the presence of the antigen, sera produced in rabbits against *M. avium* strains designated P-55 and Dent fixed the complement even in a dilution as high as 1 : 640. These sera contained also precipitins to *M. paratuberculosis*, which formed band within 24 h.

The present results are consistent with the already known fact that *M. avium* and *M. paratuberculosis* bacteria have several antigenic components in common. Of the different *M. avium* strains, strains P-55 and Dent seem to possess the largest number of antigenic mosaics identical with, or serologically very similar to, *M. paratuberculosis*.

On the basis of these findings the authors consider justified the practice that the 1 : 10 dilution of sera is used in screening for *M. paratuberculosis* infection, since thus cross. reactions arising from other mycobacterial infections can, to high probability, be eliminated.

Keywords. *Mycobacterium avium*, *Mycobacterium paratuberculosis*, complement fixation test, antigenic relationship.

In the serodiagnosis of paratuberculosis, a cellular (Gorrie, 1959) or phenol-treated (Annau, 1956) antigen prepared from *M. avium*, or a soluble antigen prepared from *M. paratuberculosis* bacteria by methanol extraction (Morris and Stevens, 1977) have been used in different laboratories.

Neither laboratory diagnosticians nor antigen producers claim that the antigens prepared in the above manner give species-specific reaction in the detection of paratuberculosis (Annau, 1956; Merkal, 1970). On the basis of Tuboly's (1965) investigations, common antigenic mosaics are held responsible for the cross-reactions (Taylor et al., 1981).

Since after infection by different *Mycobacterium* species, among them *M. avium*, positivity of serological tests aimed at demonstrating *M. paratuberculosis* can be reckoned with, we tested 45 rabbit antisera, representing 19 serotypes of Schaefer, against *M. paratuberculosis* antigen in complement fixation and gel precipitation tests to detect the possible cross-reactions.

These investigations were considered justified, since so far the cross-reactions occurring between the different *M. avium* and *M. paratuberculosis* strains

have been confirmed only by studies of the antigenic structure. The practical importance of the present work is that it can serve as a basis for a more reliable application of the complement fixation and gel precipitation tests in the serodiagnosis of paratuberculosis.

Materials and methods

Antigens

The complement-fixing antigen was prepared from the *M. paratuberculosis* strain 5889 (Bergey, 1923) obtained from Prague, according to the method of Gorrie (1959).

The gel-precipitation antigen was prepared from the same strain, by filtering 4-week Sauton broth cultures through Seitz filters and condensing the filtrate to 6.93 mg/ml dry matter content.

Test sera

M. avium antisera were produced using *M. avium* reference strains specified for this purpose (Schaefer, 1965; Viallier et al., 1977), according to the method described by Schaefer (1965). Sera of 45 immunized rabbits and a negative control serum obtained from an uninoculated rabbit were used in the determination of the 19 serotypes. The homologous titre of the sera produced in rabbits against *M. avium* was determined by the agglutination method of Schaefer (1965). The designation and serotype of the different *M. avium* strains and the homologous titres of sera produced against them are shown in Table I.

Complement fixation test

In the main experiment, 1% more complement was added to the system than that determined by the complement titration. The sera to be tested were diluted 1 : 10 and inactivated at 58 °C for 30 min. The complement fixation test was done in a water-bath of 37 °C for 2×20 min. The sera giving complete complement fixation in the 1 : 10 dilution were considered positive.

Gel precipitation test

The test was performed in 0.8% agarose prepared in Sørensen's 0.15 M phosphate buffer of pH 7.0. In the double radial gel diffusion test the volume of the wells was 0.075 cm^3. The reactions were read daily for 7 days.

Results

The results are shown in Table I.

Table I

Testing of rabbit sera produced against Schaefer's *M. avium* serotypes for paratuberculosis

M. avium serotype	Designation of strain	Homologous agglutination titres, reciprocals	With *M. paratuberculosis* antigen: Complement-fixing titre, reciprocals	Gel precipitation as read on days						
				1	2	3	4	5	6	7
1	16 909-338	80	20	—	—	—	—	—	—	—
1	16 909-338	160	320	—	—	—	—	+	+	+
1	J-2085	160	20	—	—	—	—	+	+	+
1	J-2085	160	20	—	—	—	—	+	+	+
2	14 141-1395	320	20	—	—	—	—	—	—	—
2	14 141-1395	320	40	—	—	—	—	—	—	—
2	17 752-372	320	20	—	—	—	—	—	—	—
2	17 752-372	160	10	—	—	—	—	—	—	—
3	6 197	320	10	—	—	—	—	—	—	—
3	6 197	320	10	—	—	—	—	—	—	—
4	13 528-1071	40	20	—	—	—	—	—	—	+
4	13 528-1071	160	20	—	—	—	—	+	+	+
4	P-55	20	640	+	+	+	+	+	+	+
4	P-55	20	320	+	+	+	+	+	+	+
5	4 443-1237	160	10	—	—	—	—	+	+	+
5	4 443-1237	160	10	—	—	—	—	—	—	—
6	3 454	20	—	—	—	—	—	—	—	—
6	3 454	80	40	—	—	—	—	—	—	—
6	3 454	20	—	—	—	—	—	—	—	—
7	P-49	320	20	—	—	—	—	—	—	—
7	P-49	320	80	—	—	—	—	+	+	+
7	P-49	160	10	—	—	—	—	—	—	—
8	—	—	—							
9	12 303-406	10	10	—	—	—	—	—	—	—
10	290-152	40	—	—	—	—	—	—	—	—
10	290-152	20	—	—	—	—	—	—	—	—
10	290-152	40	10	—	—	—	—	—	—	—
11	14 186-1424	40	40	—	—	—	—	—	—	—
11	14 186-1424	40	40	—	—	—	—	—	—	—
12	P-42	60	80	—	—	—	—	—	—	+
12	P-42	40	320	—	—	—	—	—	—	—
13	Chance	320	160	—	—	—	—	—	—	—
13	Chance	320	160	—	—	—	—	—	—	—
14	P-39	80	320	—	—	—	—	+	+	+
15	Dent	20	320	—	—	—	+	+	+	+
15	Dent	40	640	+	+	+	+	+	+	+
16	Yandle	160	20	—	—	—	—	+	+	+
16	Yandle	320	80	—	—	—	—	+	+	+
17	P-54	10	10	—	—	—	—	—	—	—
17	P-54	10	10	—	—	—	—	—	—	—
18	Altman	40	160	—	—	—	—	—	—	—
19	Mc Anemy	80	—	—	—	—	—	—	—	—
19	Mc Anemy	80	40	—	—	—	—	—	—	—
20	Newberry	20	40	—	—	—	—	—	—	—
20	Newberry	40	10	—	—	—	—	—	—	—
20	Newberry	40	10	—	—	—	—	—	—	—
Negative control rabbit serum		—	—	—	—	—	—	—	—	—

Discussion

In spite of the identical density of the bacterium suspensions used for immunization and the identical immunization method, rabbit sera produced against *M. avium* strains of different serotypes showed diverse homologous agglutination titres.

Of the 45 sera, 40 gave positive reaction with *M. paratuberculosis* antigen in the complement fixation test; two of the rabbits immunized against each of serotypes 6 and 10 and one of those immunized with serotype 19 proved negative in this test.

Despite the low homologous agglutination titres, anti-P-55 (serotype 4) and anti-Dent (serotype 15) sera fixed complement even in the 1 : 320 and 1 : 640 dilutions in the presence of the *M. paratuberculosis* antigen, and contained detectable quantities of anti-*M. paratuberculosis* precipitin already from the 1st day onwards. Both anti-Dent sera gave at least 2 precipitation bands.

Anti-P-55 and anti-Dent rabbit sera precipitated the *M. paratuberculosis* antigen already after 24 h; of the other sera, serotype 1, 14, 15 and 16 sera gave reaction with the same antigen as late as on the 4th or 5th day.

The present investigations are not sufficient to allow immunological interpretation of the observed phenomena. It is most likely that the P-55 and Dent strains are the most similar to *M. paratuberculosis* in antigenic structure. Namely, of the reference strains of *M. avium* two strains (P-55 and Dent) induced an antibody production resulting in an especially high complement fixation titre with the *M. paratuberculosis* antigen. It seems to be obvious that the serodiagnostic reactions are cross-reactions and can be considered specific at most for mycobacteria. However, the interpretation of the cross-reactions is further disturbed by possible *Corynebacterium renale* infections (Wilks et al., 1981).

As indicated by the results shown in Table I, according to which certain *M. avium* strains seem to be antigenically related to *M. paratuberculosis*, we consider justified that in the serodiagnosis of paratuberculosis the 1 : 8 or the 1 : 10 serum dilution is regarded as the threshold of positivity in the complement fixation test (Goudswaard et al., 1976; Trigo, 1979; Lisle et al., 1980).

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AN OUTBREAK OF MOREL'S DISEASE (A CONTAGIOUS SHEEP DISEASE ACCOMPANIED BY ABSCESS FORMATION) IN HUNGARY

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(Received October 14, 1983)

A disease characterized by suppurative lymphadenitis and subcutaneous abscess formation was observed in a sheep flock imported from France. All sheep of the flock consisting of 381 ewe hoggets fell ill within a few months. Relapse was of common occurrence. Six to eight weeks after the rupture and healing of the first abscess a second, and in some cases even a third, abscess developed. The outbreak lasted 12–13 months in the flock, then it gradually subsided and disappeared among adult sheep, but subsequently reappeared in lambs born in the meantime.

Six diseased sheep, and pus samples taken from 13 diseased animals, were examined in the laboratory. All samples yielded a slowly growing, micro-aerophil, catalase-negative, Gram-positive coccus with high nutrient requirement.

Merino sheep infected with the isolates developed subcutaneous and lymphatic abscesses similar to those found in natural cases.

Based upon the detailed examination the isolates proved to be *Micrococcus (Peptococcus) abscedens ovis*. The disease was identified as Morel's disease. Dissemination of Morel's disease in Hungary was prevented by stamping out the affected flock.

Keywords. Morel's disease, sheep, *Micrococcus abscedens ovis*, outbreak, Hungary.

In sheep, processes characterized by suppurative inflammation are most frequently caused by streptococci, staphylococci and corynebacteria. In a minority of cases other bacteria, e.g. *Pasteurella*, *Moraxella*, *Proteus*, *Peptostreptococcus*, *Pseudomonas* strains can also be isolated from abscesses (Richard et al., 1979).

Of the diseases characterized by abscess formation, caseous lymphadenitis and Morel's disease deserve interest because of their contagious nature. While numerous papers have dealt with caseous lymphadenitis, only a few reports are available in the literature on Morel's disease.

Morel (1911) had described in France a sheep disease characterized by abscess formation. The disease was named after him later on. Only French publications appeared on Morel's disease (Aynaud, 1923; Carré, 1923; Joubert, 1958) until Shirlaw and Ashford (1962) observed its occurrence in Kenya. Later, the disease was reported in Iran (Afnan and Hedjazi, 1978).

An outbreak of Morel's disease in Hungary is reported in the present paper.

Materials and methods

Six sheep showing clinical symptoms of suppurative lymphadenitis, and pus samples taken aseptically from 13 diseased sheep, were submitted for laboratory examination. The sheep were subjected to detailed gross-pathological, bacteriological, mycological and virological examination; the pus samples were examined bacteriologically.

For the mycological examinations, Czapek's agar and beer agar, for the bacteriological examinations common nutrient agar and blood agar were used. The plates were incubated at 37 °C under aerobic conditions, under 10% CO_2 and anaerobically. Characterization of the isolates was performed as described in bacteriological manuals (Buchanan and Gibbons, 1974; Lányi, 1980).

For virus isolation, the appropriately processed cell-free supernate and cell suspension of lymph nodes were added to primary and secondary calf testicle and calf kidney monolayers and monolayer cultures prepared from the FLK lamb kidney cell line. Six consecutive blind passages were performed at 4- to 7-day intervals.

Four Merino sheep were inoculated by different routes with one of the bacterium strains isolated from the natural disease.

Results

Case history

A farm in Eastern Hungary imported 381 four to five months old Lacaune ewe hoggets and 10, approximately one year old, rams from France. In a few weeks following the arrival of the animals, several ewe hoggets showed swelling and suppurative softening of one of the superficial lymph nodes. The suppurating lymph nodes reached the size of a hen's egg or of a man's fist. The submaxillary, prescapular and subiliacal lymph nodes suppurated most frequently. All ewe hoggets fell ill and showed clinical symptoms of suppurative lymphadenitis within 4 to 5 months.

Relapses occurred very frequently. Some weeks after the abscessed lymph nodes had ruptured and healed, another lymph node, and later a third one, became affected. Thus, the outbreak lasted over one year.

In the meantime the ewe hoggets reached maturity and started lambing. The disease occurred in nearly all of the lambs, a few weeks after birth. However, no mortality directly attributable to the disease characterized by abscess formation was observed.

In several cases abscess was developed in the subcutaneous connective tissue and not in the lymph nodes.

Gross-pathological findings

At autopsy, two sheep showed suppuration of the prescapular lymph node. In one case the submaxillary, in another the supramammary lymph node suppurated while in a third case suppuration of the subiliacal lymph node was associated with a pulmonary abscess. One animal exhibited a subcutaneous abscess surrounded by a capsule of connective tissue in the cervical region.

Microbiological findings

The virological and the mycological tests gave negative results.

The bacteriological examinations showed the spleen and kidney samples sterile. A Gram-positive coccus was isolated in pure culture from all the purulent lymph nodes, subcutaneous abscesses and pus samples. The main properties of this bacterium are the following. In stained smears prepared from pus samples, it occurred most frequently in triad or tetrad forms, less frequently in diplococcus form, occasionally in larger conglomerates. The bacterium has high nutrient requirement: it does not grow on common agar plates, but grows well on blood agar. In serum-containing broth the bacterium forms a granular sediment on the bottom of the tube. The organism is micro-aerophil: under aerobic conditions its pinprick-sized colonies appear on the 5th day of incubation or still later. Under 10 per cent CO_2 or under anaerobic conditions its small, off-white colonies can be observed easily already in the 48th h. In blood agar the organism causes β-haemolysis, most expressed under aerobic conditions. The bacterium is oxidase- and catalase-negative, it does not produce urease enzyme and does not liquefy gelatine. In addition to glucose, it decomposes sucrose, maltose, fructose, dextrin and trehalose with acid formation. It gives no reaction with mannitol, galactose, sorbitol, inositol and lactose.

From the pulmonary abscesses *Corynebacterium pyogenes* and *Pasteurella multocida* were isolated in mixed culture, in addition to the bacterium characterized above.

Experimental infection of sheep

Infection experiments were performed to elucidate the pathological role of the bacterium species isolated in pure culture from the abscessed lymph nodes and subcutaneous abscesses. Four Merino sheep of different age were inoculated.

A strain isolated from one of the natural cases was grown on blood agar plates, then the cultures were washed down with 0.9% saline.

The inoculum was rubbed into the scarified skin of two sheep. The third sheep was inoculated into the thigh muscle, the fourth intravenously.

The animals were killed by bleeding 3 months after infection, and were subjected to pathological and bacteriological examinations.

At autopsy, in accordance with natural cases, two sheep showed suppurative lymphadenitis (involving the prescapular and subiliacal lymph nodes, respectively), while the others developed subcutaneous abscess. One of the latter two sheep had multiple pulmonary abscesses surrounded by a capsule of connective tissue.

The Gram-positive coccus was reisolated in pure culture from all abscesses.

Discussion

Our detailed bacteriological examinations have convinced us that the bacterium species isolated from the abscesses is *Micrococcus abscedens ovis*, while the disease caused by it is Morel's disease, which had not occurred in Hungary before.

Of the properties of this organism, its typical micro-aerophil nature, high nutrient requirement, slow growth, its characteristic arrangement in pus, granular growth in broth, oxidase-, catalase- and urease-negativity, glucose-fermentation and specific pathogenicity to sheep have to be stressed. Based upon these properties, the organism can be distinguished from the *Streptococcus* spp. of veterinary importance, as well as from *Staphylococcus*, *Micrococcus*, *Peptococcus*, *Peptostreptococcus* and *Sarcina* spp. It can be discerned from *Corynebacterium ovis* already on morphological grounds.

In our opinion, the organism, being catalase-negative, cannot be classified as a member of the *Micrococcus* genus. In the 8th edition of Bergey's Manual of Determinative Bacteriology (1974), it is listed among members of the *Peptococcus* genus, not among those of *Micrococcus*.

Between the micrococci described by different authors in connection with Morel's disease, there are differences as regards biochemical properties. Our strains also differ from the strains described by foreign authors, primarily in respect of carbohydrate-decomposition. Probably *Micrococcus abscedens ovis* has several biotypes, but the involvement of different *Micrococcus* species in Morel's disease cannot be excluded either. Further studies are needed to determine the exact taxonomic place of the organism.

Considering these still unsolved problems, Morel's disease can be defined as an endemic sheep disease accompanied by a characteristic abscess formation, the causative agent of which has the properties described above. On the basis of these properties, the organism can be distinguished from the known *Streptococcus* spp. and also from the other Gram-positive cocci.

Joubert (1958) reported the non-immunogenic nature of *Micrococcus abscedens ovis*. The fact that animals affected by the outbreak under study

developed abscesses repeatedly, is in accordance with this. However, the outbreak gradually subsided and the disease disappeared from the adult flock, suggesting that, although very slowly, a weak immunity still developed after infection. This course of epizootic cannot be explained by the development of an age-dependent resistance, since in the infection experiments the susceptibility of sheep appeared to be independent of age.

During the outbreak, superficial lymph nodes became suppurated and abscesses developed in the subcutaneous connective tissue; from other organs the causative agent was isolated only once (from the lungs of an animal). On the other hand, severe lung lesions developed in an animal infected intravenously. It seems that such disease processes have to be reckoned with only in case of extremely severe infection or if the resistance of the animal has been impaired by stress factors.

Morel's disease of sheep had not been diagnosed in Hungary previously. Although fatal cases occur only exceptionally, prevalence of the disease in this country would make marketing of mutton almost impossible and would throw difficulties in the way of milk marketing (in several animals the supramammary lymph nodes became affected). It should be noted that the Merino breed widely used in Hungary is as susceptible to the disease as the Lacaune breed in which the disease was diagnosed. To avoid dissemination of the disease, we recommended to stamp out the affected sheep flock, which had been kept in isolated premises throughout.

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AMINO ACID COMPOSITION AND CHARACTERISTIC INDICES OF BOVINE SEMINAL PLASMA IN CASE OF INFECTION OF SEMEN BY MYCOPLASMAS

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Free amino acid and total protein content as well as laboratory indices (ejaculate volume, pH, number of spermatozoa per unit volume, motility, motility after deep-freezing for 24 h and after rethawing, and fertility %, i.e. the number of cows that did not return to oestrus within 3 months) of seminal plasma from 10 healthy bulls and 10 bulls infected by *Mycoplasma bovis* or *Ureaplasma* were determined. As a rule, the seminal plasma of healthy bulls had higher free amino acid mean values, viz., the mean value of total free amino acids was 1.988 ± 0.850 g/l and 2.671 ± 1.190 g/l in mycoplasma-infected and mycoplasma-negative bulls, respectively. Owing to the considerable deviation and the low number of bulls, significant differences between mycoplasma-infected and healthy bulls were found only in respect of three parameters, viz. the glutamic acid content, a parameter characteristic of semen quality (0.634 ± 0.430 and 1.239 ± 0.699 g/l; $P < 0.05$), the tyrosine content, and the total protein content (62 ± 9.0 and 52 ± 1.2 g/l; $P < 0.05$). Ejaculate volume, motility of spermatozoa, and results of the deep-freezing test appeared to be more favourable for the healthy bulls, however, the differences were not significant. In the authors' opinion, changes in the amino acid content depend on the degree of infection and on the severity of tissue changes. This means that the amino acid content of the seminal plasma may be a valuable indicator of semen quality.

Keywords. Seminal plasma, mycoplasma, free amino acids, bull, semen quality.

The last decade has seen a considerable increase of interest in mycoplasmas. This can be attributed, among others, to the fact that a large group of diseases can be traced back to mycoplasma infection. Blanchard and Garcia (1981) considered the mycoplasma- and other bacterial infections of semen cultures and of the genital tract to be an important factor of infertility. Mycoplasmas have frequently been isolated from genital organs of bulls (Ernø, 1974; Jasper et al., 1974; Onovarian et al., 1975), and, naturally, also from semen. In the vast majority of mycoplasma isolations, clinically apparent disease could not be observed. On the other hand, some authors bring the chronic vesiculitis and epididymitis of bulls (Al-Aubaidi et al., 1972; Ernø, 1974), poor motility and viability of spermatozoa, with special regard to freezability (Hall and McEntee, 1981; Jurmanova and Sterbova, 1977), and also infertility (Tourtellotte and Lein, 1976) into causal relationship with mycoplasma infection. The

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microbiological examinations have confirmed the occurrence of several *Mycoplasma* species in semen. *Mycoplasma bovigenitalium* and *M. bovis*, and *Ureaplasma* spp. have been isolated from semen most frequently (Ernø, 1974; Hirt et al., 1967; Jurmanova and Sterbova, 1977), but isolation of *Acholeplasma laidlawii* and *M. arginini* has also been reported (Leach, 1970; Cottew and Leach, 1969).

According to data of Ibrahim and Boldizsár (1980), the free amino acid content of seminal plasma from healthy bulls of excellent fertility is higher, while its protein content lower, than the same parameters of the poor-quality seminal plasma from bulls of low fertility.

The aim of the present work was to compare the free amino acid content and characteristic indices of bovine seminal plasma from mycoplasma-infected and mycoplasma-negative semen.

Materials and methods

Semen samples were collected from 4 years old Hungarian Fleckvieh bulls at the Central Hungarian Artificial Insemination Station. The volume, pH, cell count of the samples, and motility, motility after deep-freezing for 24 h and rethawing, and the fertility % (the proportion of cows that do not return to oestrus within 3 months) were determined. Total protein content was determined as described earlier (Ibrahim and Boldizsár, 1980).

All samples were divided into two parts; one part was used for mycoplasma isolation. For the isolation of microorganisms belonging to the *Mycoplasma* genus, nutrient media designated "B" (Ernø and Stipkovits, 1973), while for *Ureaplasma* isolation "LSB" media (Livingston, 1972) were inoculated with the samples. Subsequently, serial dilutions between 1 : 10 and 1 : 1000 were made, and the inoculated media were incubated at 37 °C for 6 days. On post-inoculation days 2, 4 and 6, inoculated nutrient broth samples were transferred to solid media, and the agar plates were incubated at 37 °C in the presence of 5% CO_2. Mycoplasma growth was checked every two days by microscopy. In case of mycoplasma growth, the isolate was identified on the basis of culturing properties, biochemical reactions and serological properties (growth-inhibition test) as described earlier (Stipkovits, 1973).

To determine the free amino acid content, the remaining part of the semen sample was centrifuged, the cell content of the plasma was examined by microscopy, and the sample was immediately deproteinized by addition of the same volume of 20% sulphosalicylic acid, to prevent peptidase activity. After repeated centrifugation, 0.5 ml sample (0.1—0.5 μmol/component) was applied on a Biocal BC-200 automatic amino acid analyser column. A two-column procedure was used, with Aminex-5 and Aminex-6 resins. Elution was performed using

citrate buffers of pH 3.25, 4.25, and 5.25. A norleucine internal standard was used. The *Glu* and *Glu*-NH_2, as well as the *Asp* and *Asp*-NH_2 values are given together. The values were determined on the basis of a curve obtained using a standard amino acid mixture of known volume.

Results

The most frequently isolated strains proved to be *M. bovis* and *Ureaplasma* spp. Of the 181 samples examined earlier, 26.5% yielded *M. bovis*, and 10.5% *M. bovis* and *Ureaplasma* (Stipkovits, unpublished results). In the present work, 10 of the examined 26 samples proved to be infected. The free amino acid content of seminal plasma from 10 infected bulls, selected on the basis of mycoplasma isolation results, and from 10 uninfected bulls serving as control is shown in Fig. 1. Values of the individual amino acids showed considerable variation. The mean values for mycoplasma-free samples were generally higher than those obtained for the infected samples. The seminal plasma of healthy bulls had higher *Asp*, *Thr*, *Ser*, *Glu*, *Ile*, *Tyr* and *Arg* values, but lower ammonia level.

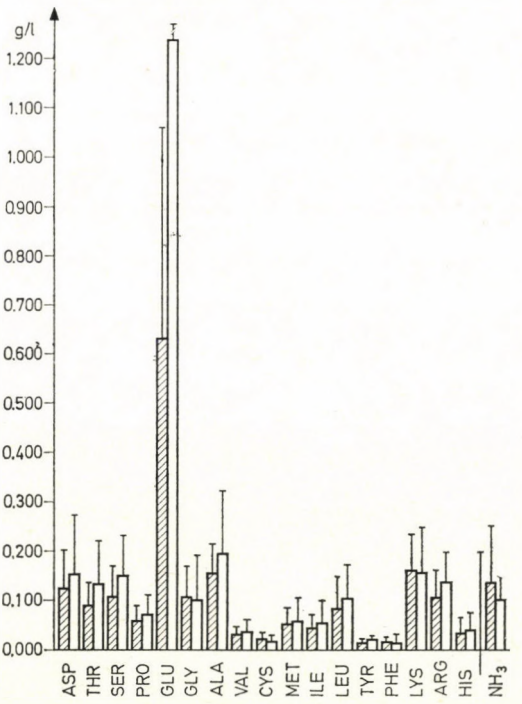

Fig. 1. Free amino acid content of the seminal plasma. Striated columns: mycoplasma-infected semen; white columns: semen of healthy bulls

The total free amino acid content was also higher in seminal plasma from healthy bulls. Because of the low number of bulls and the high standard deviation (SD), the differences were statistically nonsignificant, except for the *Glu* and *Tyr* values ($P < 0.05$). Glutamic acid is a decisive constituent of the seminal plasma, and its concentration is a characteristic index of semen quality (Horváth and Solymoss, 1954; Ibrahim and Boldizsár, 1980).

Total protein content was significantly lower in uninfected semen samples (Table I). Other indices characteristic of semen quality (especially ejaculate

Table I

Laboratory parameters of the semen and fertility
(n = 10)

Parameter	Positive	Negative
Total protein, g/100 ml	6.2±0.9	5.2±1.2
Semen volume, ml	6.5±1.7	7.2±1.9
Sperm count, $\times 10^9$/ml	1.6±—	1.8±0.3
Motility, %	66.5±4.1	70.5±3.6
Resistance test, %	38.0±2.5	42.0±3.0
Resistance test, 24 h after deep-freezing	29.0±3.2	35.5±2.0
Fertility, %	46.0±—	48.0±—

volume, motility of spermatozoa, and results of the deep-freezing resistance test) were also more favourable in semen free from mycoplasma, but the difference was not statistically significant. The chromatogram of the seminal plasma had 6 large, unidentified, peaks (mainly before the acidic amino acids, corresponding to minutes 17, 21, 28, 35, 37 and 46 in a run of 150 min), and 12 additional small peaks between the acidic-neutral and basic amino acids. These may represent short-chain peptides, amino acid derivatives or other ninhydrin-positive substances as well.

Discussion

The values found by us for the seminal plasma of healthy bulls are in good agreement with earlier results obtained for bulls of varying reproductive performance (Ibrahim and Boldizsár, 1980). Changes in the concentration of amino acids between the head and tail of the epididymis vary between species. The difference of the amino acid concentration of the epididymal plasma from that of the rete testis fluid and of the seminal plasma can be explained by the fluid resorption taking place in this area (Brown et al., 1972; Johanson et al., 1972;

White et al., 1972). In addition, certain amino acids mentioned above are present in higher concentrations in the seminal plasma of bulls with higher fertility (Ibrahim and Boldizsár, 1980). The latter observation indicates that fluid resorption is not the only factor determining the amino acid composition of the seminal plasma. It is known that different parts of the genital tract vary in their ability to secrete amino acids from the blood stream into the lumen, which, presumably, provides optimal conditions for the survival of the sperm cell (Huang and Johnson, 1975). Since spermatozoa become fully mature only after the days spent in the epididymis, new knowledge concerning the free amino acid content may contribute to a better understanding of the maturation mechanism of spermatozoa.

The transportation activity of cells of the genital tract can be influenced by several factors. Adverse environmental conditions, the manner of sexual preparation, the weekly number of ejaculates, hormonal effects and several others factors may influence the absorptive activity and amino acid secretion of cells of the genital tract, and may alter the rate of transport. Our results allow us to conclude that mycoplasma infection is one of these factors. Presumably, the amino acid content depends on the degree of mycoplasma infection and on the severity of cellular changes induced by mycoplasmas. Accordingly, the amino acid content of the seminal plasma can be a useful index in semen qualification.

The results of our investigations show good agreement with data of the literature. The obtained results can be easily explained by mycoplasma infection of the semen. In mycoplasma infection spermatogenesis is usually normal (Parsonson, 1970), as it was found also by us. The mycoplasmas adhere to epithelial cells, and by competitive utilization of their enzymes, toxins and other important substances (e.g. amino acids, nucleic acids, precursors) alter the metabolism of host cells, occasionally leading to cell death (Barile, 1979; Fogh, 1973). This results in the initiation of a more or less severe inflammatory process, which brings about an increase in the protein content of the seminal plasma in the first stage. In more severe cases the glandular epithelium undergoes histological changes or the glands become enlarged and pus cells appear in the semen (Ernø, 1974). Adherence of mycoplasmas to spermatozoa (Holzman et al., 1978; Panangala et al., 1981) may in itself lead to a decreased motility of spermatozoa (Jurmanova and Sterbova, 1977). A linear relationship exists between the viable mycoplasma concentration present and the decrease of spermatozoal motility.

Mycoplasma infection has been reported to increase the aspartate-transaminase (AST) activity in tissue (Hilmy, 1983), which may result in a decrease of glutamic acid concentration.

Concentrations of the individual amino acids showed considerable variation. The most characteristic amino acid constituent of semen was glutamic

acid, which represented the largest proportion of all amino acids. The concentration of *Glu* was 0.634 ± 0.430 and 1.239 ± 0.699 g/l in the mycoplasma-positive and mycoplasma-negative group, respectively. This can be explained only partly by the relatively low number of bulls (the number of bulls was limited because of the low number of positive animals available); the individual variation is also considered important. Taking into account the fact that earlier 0.240 ± 0.049 and 1.043 ± 0.662 g/l *Glu* concentrations had been obtained for bulls of poor and good reproductive performance, respectively (Ibrahim and Boldizsár, 1980), the role of further factors has to be considered, among others the seasonal fluctuation of sex hormone levels in the blood. In ruminants, testosterone concentration shows a considerable seasonal fluctuation (Lincoln, 1981). According to the investigations of Horváth and Solymoss (1954), the amino acid content of the seminal plasma is under testosterone regulation, thus the fluctuation of its level may depend also on hormone secretion.

In the present work ureaplasmas were also isolated from the semen. Ureaplasmas split urea, thus, the increased ammonia concentration in the seminal plasma of some of the infected bulls is easy to explain. Increased ammonia concentration exerts an adverse effect on the motility and viability of spermatozoa. Finally, mycoplasmas are able to take up amino acids (alanine, aspartic acid, glutamic acid, etc.), and their metabolism associated with oxygen uptake is enhanced in the presence of amino acids (Rodwell, 1969; Schimmel, 1963). Presumably this is why the quantity of certain amino acids (e.g. glutamic acid) is significantly decreased in mycoplasma-containing semen. Furthermore, an altered resorptive function of mycoplasma-infected epithelial cells may also have led to changes in the amino acid composition of the seminal plasma. Insufficient concentration of certain amino acids in the seminal plasma can also affect the viability of spermatozoa adversely. Thus, mycoplasma infection, sooner or later, leads to infertility. In addition to this, the risk has to be reckoned with that with mycoplasma-containing semen mycoplasma infection of poor prognosis can be spread in cow populations.

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PATHOLOGICAL STUDIES IN CHICKEN EMBRYOS AND DAY-OLD CHICKS EXPERIMENTALLY INFECTED WITH AVIAN REOVIRUS

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Fifty and 30 chicken embryos derived from an SPF flock were infected with an avian reovirus strain on the 8th (A) and 14th (B) day of incubation, respectively; 20 chicks (C) were infected on the 1st day after hatching. Three birds were taken from each group at regular intervals after infection, and their organs were studied by morphological methods. Results of earlier studies performed on healthy embryos and chicks were used for comparison. Attempts were made to reisolate the virus. The immunorosette-formation and lymphocyte-stimulation tests were performed with lymphocytes obtained from the blood, thymus and bursa of Fabricius on the 14th and 20th days of incubation and on the 20th day after hatching.

Chicken embryos of different age and day-old chicks responded to reovirus infection differently. In group A, mortality, retarded development, and in different organs exudative inflammation accompanied by signs of circulatory disorder, degenerative changes (cell degeneration and necrosis) and syncytium formation were observed. The separated lymphocytes failed to respond to antigenic stimulus in vitro.

In group B and group C no mortality, in group C even no clinically apparent disease occurred. However, the chicks developed unevenly. In some organs (liver, pancreas, myocardium and glandular stomach wall) productive, focal inflammatory-cellular infiltrations were seen, similar to those often found during diagnostic examination of 2 to 4 weeks old chickens infected by reovirus. A part (5–30%) of the lymphocytes obtained from these birds gave in vitro rosette-formation with reovirus-infected erythrocytes, and 10–25% of them responded to antigenic stimulus with blastogenic transformation.

Keywords. Experimental infection, immune system, chicken embryo, day-old chick, pathology.

The judgement of the primary pathogenic role of avian reoviruses lacks uniformity. Several authors consider proved their primary role in producing certain diseases (arthritis and tenosynovitis of 2–4 weeks old chicks, myocarditis, hydropericardium and the so-called hepato-myelopoietic disease), others question the existence of such a role and regard other, nonspecific, factors as being of decisive importance. It is supposed that reovirus infection plays a role in suppressing the immune response of birds, thereby influencing the success of vaccinations against other infectious diseases, and that by decreasing the resistance of birds it contributes to the development of infectious bursal disease (Gumboro disease) and inclusion-body hepatitis (Kudron et al., 1982, 1984). Menendez et al. (1975) found that in embryonated eggs infected with a low-titre virus part of the embryos died between the 10th and 18th days of incubation; other embryos hatched with a decreased vitality.

Our studies on the pathogenic effects of avian reovirus on chicken embryos and day-old chicks are reported in the present paper. A further aim of the present work was to obtain information on the cellular reactions of chicken embryos of different age and on the development of their cellular responsiveness demonstrable in vitro.

Materials and methods

One hundred and twenty dwarf Leghorn chicken embryos and 50 chicks coming from an SPF flock were used. The eggs were incubated in a RAGUS-60-type laboratory incubator.

Experimental infection. Fifty and 30 embryos were inoculated into the allantoic cavity with 100 CPU of a reovirus strain isolated from the organs of a 2 weeks old chick on the 8th (group A) or 14th (group B) day of incubation; the remaining 40 embryos served as control. The eggs were candled twice daily. Subsequently, every 2 days two embryos from each group were processed for histological, while one embryo from each group for electron microscopic and virological examinations. The embryos found dead at candling were used for histological, virological, bacteriological and mycological examinations. Twenty one-day-old chicks (group C) were inoculated with the same virus dilution orally.

Two chicks were killed for the above examinations from each group on days 1, 2, 5, 10, 20 and 30 after hatching.

Histological and electron microscopic examinations. These were performed as described elsewhere (Glávits et al., 1981), with the exception that histological sampling was extended to the yolk sac wall and the chorioallantoic membrane, and, in hatched chicks, to the brain, kidneys, lungs, heart, pancreas, glandular stomach and tarsal joint in addition to the organs listed in the cited paper. For electron microscopy, samples were taken from the liver, the heart and the glandular stomach.

Virus isolation. Virus isolation was attempted in primary chicken kidney cell cultures from the allantoic fluid and organ homogenates of embryos and from spleen, liver and lung homogenates and faeces of chicks at each sampling time.

In vitro study of cellular reactions. Samples from group A were examined. Thymus and bursa of Fabricius from 7 birds and blood from 3 birds killed on each of the following occasions were used: 6th day post-infection (PI) (14th day of incubation), 12th day PI (20th day of incubation) and 33rd day PI (20th day after hatching). Blood and organ samples from uninfected (healthy) birds served as control.

Separation of lymphocytes. From 2 ml of blood samples, leucocytes were obtained by Ficoll-Paque by centrifugation (1000 rpm, 20 °C, 30 min), then

suspended in Hanks's solution containing 5% fetal calf serum. Cell density was adjusted to 10^5/ml. The cell suspension obtained from one bird was distributed in 4 Leighton tubes (0.2 ml). The bursa and the thymus were cut into small pieces, and cells were obtained by trypsin treatment. The pooled cells were washed and their suspensions were adjusted to 10^5 cells/ml.

The *lymphocyte-stimulation and immunorosette-formation tests* were performed according to the method described by Szent-Iványi et al., 1981, with the modification that the cell cultures stimulated with 4 CPU reovirus for 72 h were fixed with methanol, and then the blastogenic transformation was examined morphologically in May–Grünwald preparations. For immunorosette-formation, erythrocytes were pretreated with 1 : 125,000 tannic acid. To each cell suspension derived from individual birds, 0.4 ml erythrocytes treated and not treated with reovirus were added up to a density of 10^6/ml. Two samples each were tested with lymphocytes isolated from the blood, the thymus and the bursa of each bird.

Results

The virus was reisolated from the allantoic fluid and organs of all the killed and succumbed embryos of group A and from all the killed embryos of group B. From hatched chicks of these groups and from chicks bled on the 2nd and 10th day, the virus was reisolated from both the organs and the faeces, while from chicks bled on the 5th and 20th day only from the faeces. In group C only faecal samples yielded reovirus, between the 5th and 20th days. All attempts to isolate virus from control birds failed.

In group A the average body size, measured and calculated on the basis of the longitudinal section of embryos cut in the plane of the vertebral column, was smaller than that of the controls, from the days immediately following infection up to hatching (Fig. 1). The daily distribution of embryo mortality that occurred between the 3rd and 12th days PI is shown in Fig. 2. In organs of embryos killed or died on the 1st or 2nd day PI (i.e. on the 9th or 10th day of incubation), histological examination revealed minor haemorrhages and, in some places, oedema. Between the 4th and 8th days PI (i.e. the 12th and 16th days of incubation) the tissues and organs showed slight yellowish discolouration visible with the unaided eye. Occasionally, the marginal parts of liver lobes contained yellowish-white necroses the size of a pinhead and the shape of a cumin seed (Fig. 3).

On histological examination the succumbed embryos showed more severe lesions than the killed ones. The lesions were of similar nature. In the dilated sinusoids of the *liver* the endothelial cells became swollen, and in their lumina heterophil granulocytes, monocytes and Kupffer cells accumulated. Of these, numerous cells showed signs of degeneration or necrosis (karyopycnosis or

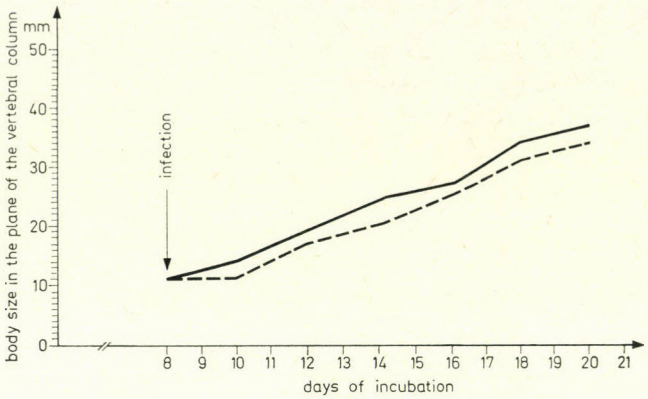

Fig. 1. Average growth rate of chicken embryos infected with reovirus (broken line, infected embryos; solid line, controls)

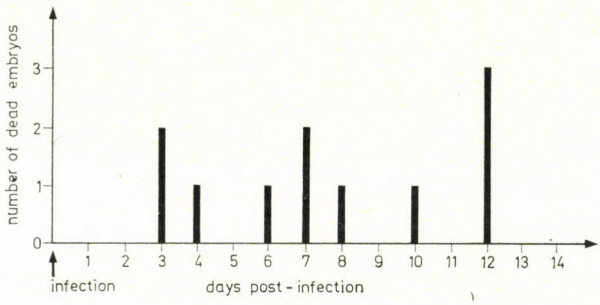

Fig. 2. Mortality of chicken embryos infected with reovirus on the 8th day of incubation

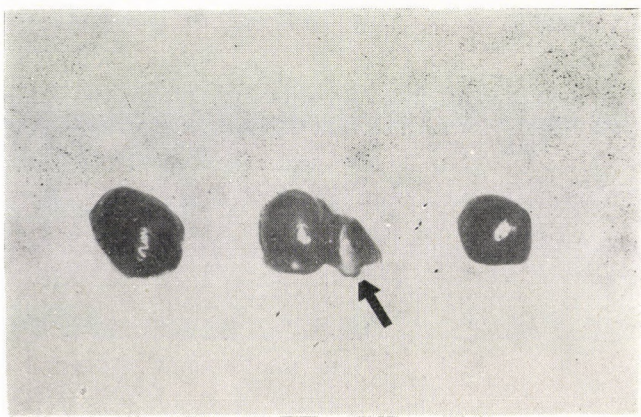

Fig. 3. Swollen livers from 12 days old chicken embryos infected with reovirus 96 h earlier (arrow indicates necrosis)

karyorrhexis). Often phagocytosis of cell debris, and multinucleated giant cells were also recognized (Fig. 4). Between the hepatocytes, bile pigment granules or cylinders, while in the parenchyma disseminated, small, circumscribed necrotic areas or larger, confluent necroses localized in the marginal parts of lobes (Fig. 5) were seen. There was a pronounced infiltration consisting mainly of heterophil granulocytes in the periportal tissues. The red pulp of the *spleen* showed signs indicative of intensive haemocytopoiesis, while the sinusoids

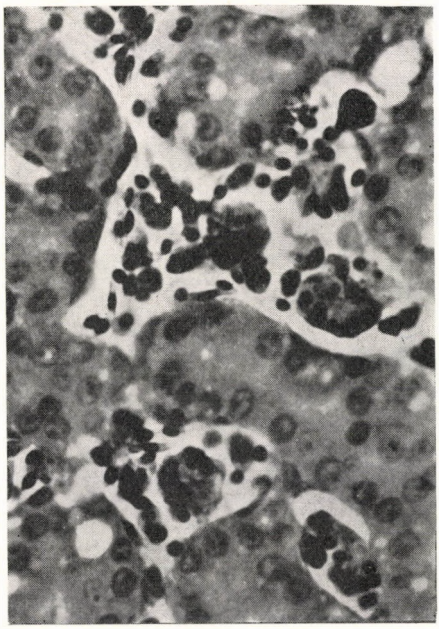

Fig. 4. Detail of the liver shown in Fig. 3 on the left. Inflammatory cells and multinucleated syncytial giant cells in the dilated sinusoid. Haematoxylin-eosin (H-E), × 400

showed changes resembling those found in the liver (cell necrosis, syncytium and giant cell formation). In the connective tissue between the developing secretory end-pieces of the *pancreas* diffuse infiltration by heterophil granulocytes, in the *myocardium* focal infiltration by similar cells (Fig. 6), and in the mucous membrane of the *glandular stomach* signs indicating intensive secretory activity of glands, degeneration and detachment of glandular epithelium as well as syncytium formation from detached epithelial cells (Fig. 7) were observed. Leucocytes representing different stages of granulocytopoiesis (myeloblasts, promyelocytes, metamyelocytes) were present in the embryonic mesenchyma of the developing bursa of Fabricius still devoid of lymphoid follicles in larger numbers and density than in the controls, while the epithelial cell groups (cor-

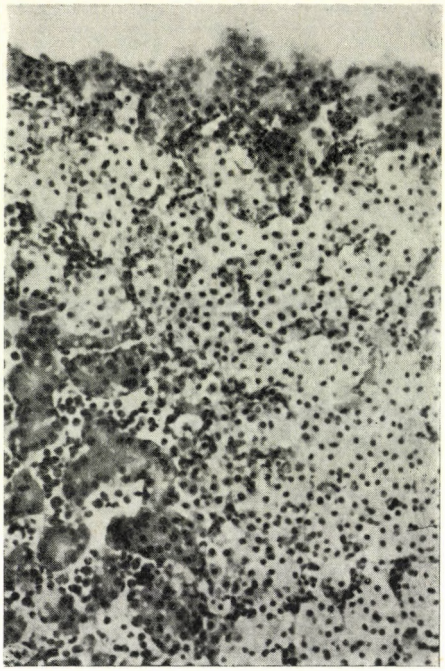

Fig. 5. Detail of the liver shown in the middle Fig. 3. Extensive necrosis of parenchyma. H-E., × 160

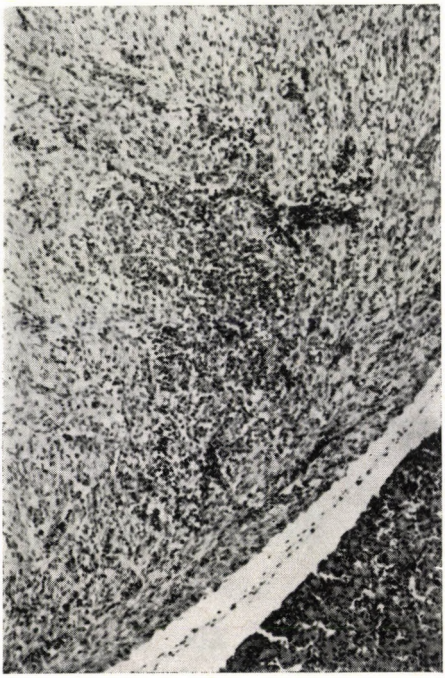

Fig. 6. Detail of heart from a 12 days old chicken embryo infected with reovirus 96 h earlier. Circumscribed infiltration with inflammatory cells in the myocardium. H-E., × 63

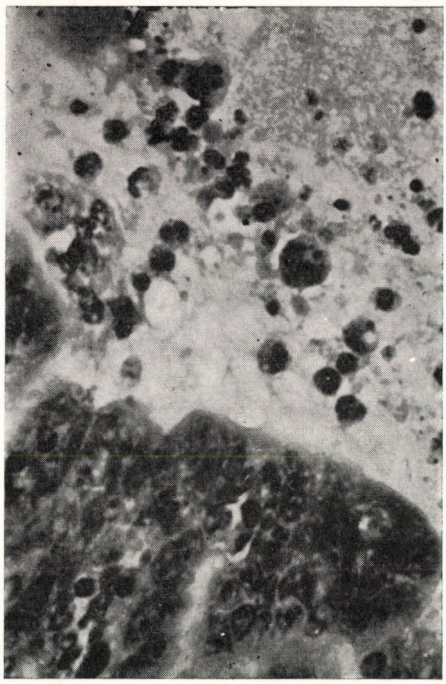

Fig. 7. Detail of glandular stomach from a 12 days old chicken embryo infected with reovirus 12 h earlier. Inflammatory cells and multinucleated syncytial giant cells in the exudate on the mucous membrane surface. H-E., × 400

responding to primordia of follicles) were smaller in size than those in the controls of the same age. There were no pathological lesions in other organs.

Between the 9th and 12th days PI (i.e. the 17th and 20th days of incubation), the liver of group-A embryos showed changes similar to those described above, with the difference that in the periportal tissues the infiltration by proliferative cells was more pronounced (Fig. 8), while the exudative changes of sinusoids and the degenerative changes of cellular elements within the sinusoids were considerably less severe. There was a demarcation zone consisting of inflammatory cells along necrotic areas of the parenchyma. In inflammatory cell groups occurring here and there, a few mononuclear cells (lymphocytes, histiocytes and plasma cells) were present among the heterophil granulocytes. The red pulp of the spleen showed a multiplex focal proliferation of reticulocytes, whereas in the mucous membrane of the glandular stomach focal infiltrations consisting of heterophil granulocytes and mononuclear cells occurred. On the other hand, at that time marked exudative changes, cell degeneration, necrosis and syncytium formation were not observed in any of the organs. The pancreas and the heart showed lesions similar to those described above, while the bursa of Fabricius exhibited disturbed development of lymphoid tissue. The rate of

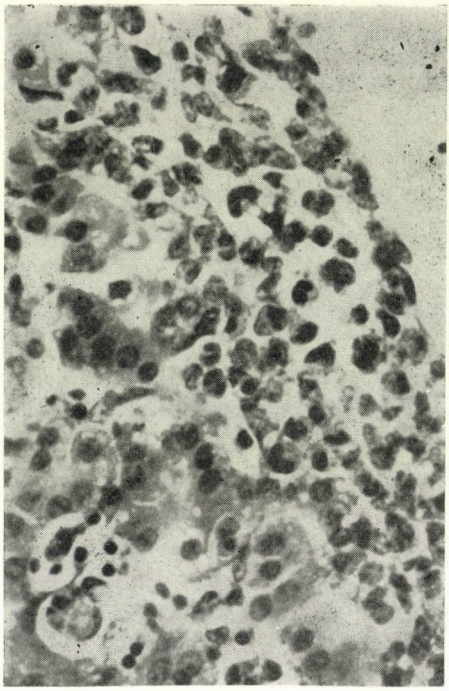

Fig. 8. Detail of liver from an 18 days old chicken embryo infected with reovirus 10 days earlier. Several rows of inflammatory cells infiltrate the interstices. H-E., × 400

colonization of lymphocytes in the developing follicles was uneven and slower than in the controls. Small follicles consisting only of reticulocytes, and large follicles rich in lymphocytes occurred simultaneously, side by side (Fig. 9). The development of lymphoid tissues in Peyer's patches located at the caecal orifice was also slower than in the controls. No appreciable changes were found in the wall of yolk sacs and chorioallantoic membranes.

No mortality occurred in group B. The embryos were not considerably smaller than the controls. The organs showed proliferative changes similar to those found in group A between the 9th and 12th days PI (i.e. 17th and 20th days of incubation). However, the lesions found in group B were somewhat more pronounced. In both group A and group B the bacteriological and mycological examination of yolk and liver samples gave negative results in both the succumbed and the killed embryos.

On days 1, 2, 5, 10 and 20 after hatching, the surviving birds of groups A and B showed a gradually progressing growth disturbance. On days 5 and 10 the average body weight, computed from the weight of 5 chicks, was lower (56.5 and 105 g, respectively) than in the control group (61.3 and 113.5 g). There were greater differences between the extreme values (44–82 g and 76–151 g) than in

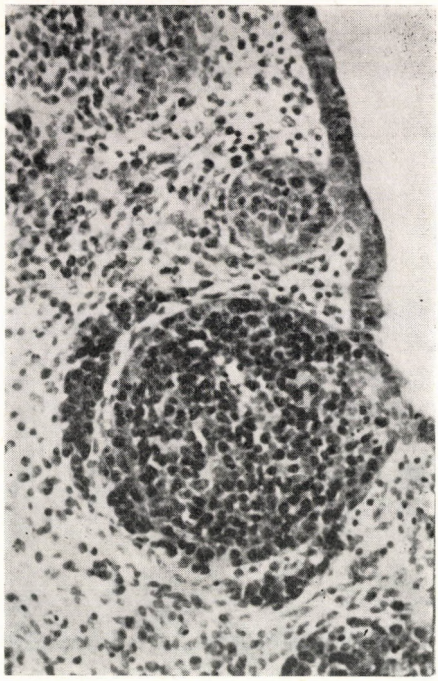

Fig. 9. Detail of bursa of Fabricius from an 18 days old chicken embryo infected with reovirus 10 days earlier. The developing lymphoid follicles differ in size and degree of lymphocyte colonization. H-E., × 160

the controls (51–69 g and 96–130 g). No mortality occurred. At necropsy of birds killed by bleeding at different times, no conspicuous pathological lesions were seen. As opposed to the controls, the histological examination of chicks of both group A and group B revealed focal inflammatory infiltrations consisting primarily of mononuclear cells (lymphocytes and, in smaller numbers, histiocytes and plasma cells) and occasionally also of heterophil granulocytes, in periportal tissues of the liver. Rarely infiltration occurred also in the parenchyma (Fig. 10), resulting in degeneration and atrophy of the affected parenchymal cells. Similar focal changes were seen in the interstices and exocrine substance of the pancreas, in the connective tissue of the deep propria glands of the glandular stomach, in the myocardium, and intralobularly in the lungs. Although colonization by lymphocytes of the bursal follicles and of Peyer's patches located at the caecal orifice took place, in the first 10 days it was slower in these groups than in the controls of the same age. No pathological lesions were observed in the brain, kidneys and tarsal joints.

The bacteriological and mycological examinations of liver and heart blood of chicks of groups A and B gave negative results in all cases.

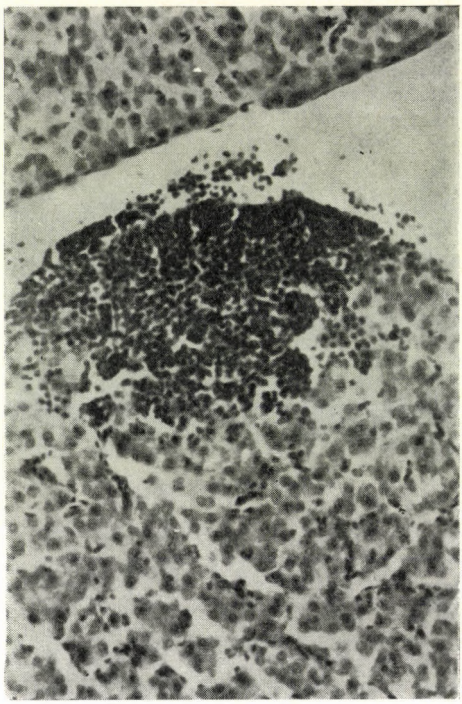

Fig. 10. Detail of liver from a 20 days old chick infected when one-day old with reovirus Circumscribed inflammatory infiltration with mononuclear cells in the parenchyma. H-E., × 160

Neither clinically apparent disease nor mortality occurred in group C. However, similarly to hatched chicks of groups A and B, chicks of group C showed uneven growth rate (disturbed growth). At necropsy of chicks bled at different times, no conspicuous pathological lesions were found.

As opposed to the controls, histological examination of different organs of group-C chicks revealed lesions similar to, sometimes more severe than, those found in hatched and survived chicks of groups A and B. The bacteriological and mycological examinations of liver and heart blood gave negative results in all cases.

By electron microscopy, virus particles corresponding to the strain used for infection were demonstrated in the liver and glandular stomach of group A embryos killed on the 4th and 6th days PI. The particles were seen singly or in small groups mostly perinuclearly, in the cyst-like dilatations of the endoplasmic reticulum of hepatocytes, endothelial cells and in epithelial cells of the mucous membrane of the glandular stomach (Fig. 11).

In in vitro study of cellular reactions, cells separated on the 6th day PI (i.e. 14th day of incubation) failed to respond to antigenic stimulus. On the 12th

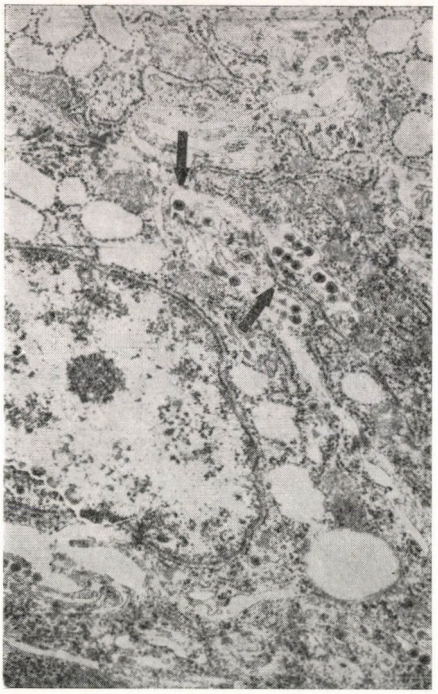

Fig 11. Detail of glandular stomach from a 12 days old chicken embryo infected with reovirus 96 h earlier. Note the perinuclear viral particles in a glandular epithelial cell (arrow). Electron micrograph, × 29,000

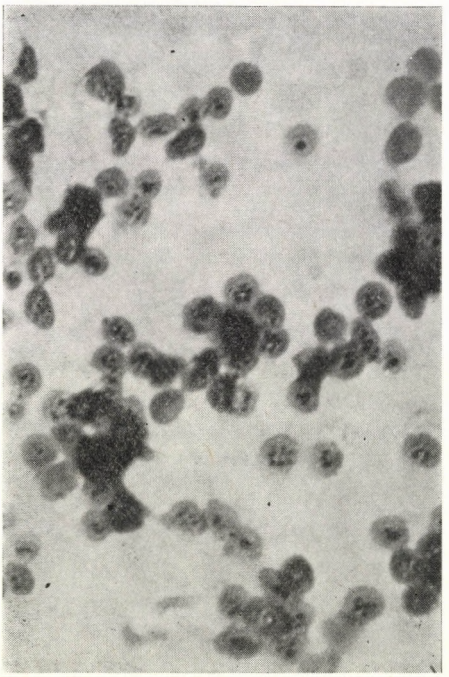

Fig. 12. Lymphocytes of a 20 days old chicken embryo infected with reovirus on the 8th day of incubation show in vitro rosette-formation with reovirus-infected sheep erythrocytes. May–Grünwald staining, × 1000

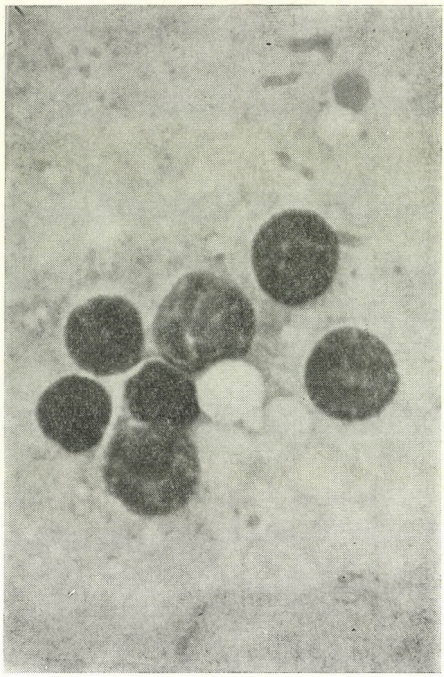

Fig. 13. Lymphocytes of a 20 days old chicken embryo infected with reovirus on the 8th day of incubation undergo blastogenic transformation after in vitro stimulation with reovirus. May–Grünwald staining, × 1000

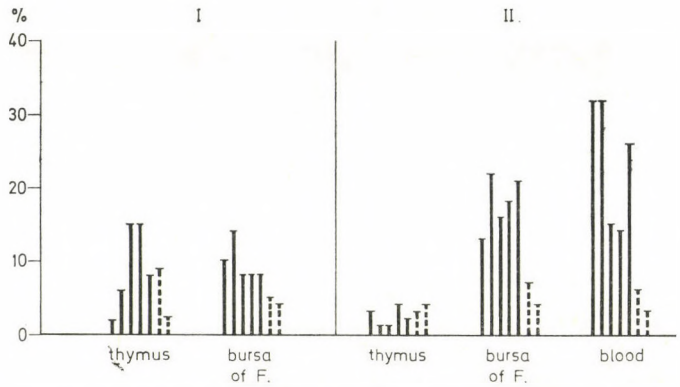

Fig. 14. Proportion of immunorosette-forming cells on the 12th (I) and 33rd (II) days PI (i.e. 20th day of incubation and 20th day after hatching, respectively). Solid line, infected birds; broken line, control birds

day PI (20th day of incubation), lymphocytes of both the infected and the control birds responded to antigenic stimulus (Figs 14 and 15). However, there was no significant difference between infected and control birds in the percentage of rosette-formation (Fig. 12) and lymphocyte-stimulation (Fig. 13). On the

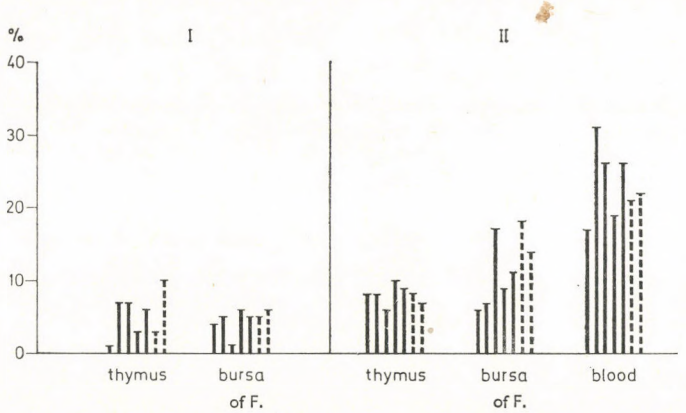

Fig. 15. Proportion of lymphocytes showing blastogenic transformation on the 12th (I) and 33rd (II) days PI (i.e. 20th day of incubation and 20th day after hatching, respectively). Solid line, infected birds; broken line, control birds

other hand, on the 33rd day PI (i.e. the 20th day after hatching) blood and bursal lymphocytes of infected birds showed a significantly higher rate of rosette-formation with reovirus-treated erythrocytes than the control lymphocytes.

Discussion

The response of the chicken embryos and day-old chicks to reovirus was age-dependent. In embryos infected on the 8th day of incubation (group A), mortality and retarded growth occurred, the organs showed signs of circulatory disturbance and exudative inflammation accompanied by cell degeneration and necrosis and syncytium formation. Virus particles could be demonstrated by electron microscopy only in this period since at that time the defensive (lymphatic) system of the chicken embryo is still undeveloped (Firth, 1977; Tizard, 1977; Glick, 1979; Glávits et al., 1981). This is in accordance with the fact that the cells separated for cellular reactions in vitro on the 14th day of incubation still failed to respond to antigenic stimulus.

No mortality occurred in the group inoculated on the 14th day of incubation (group B). The organs showed mostly productive lesions characterized by infiltration with heterophil granulocytes (and not accompanied by appreciable cell degeneration, necrosis or syncytium formation).

In group A, the lymphocytes separated for in vitro cellular reactions formed rosettes with reovirus-infected sheep erythrocytes and responded to reovirus antigen with blastogenic transformation.

In the group infected on the 1st day after hatching (group C) neither mortality nor clinically apparent disease occurred. However, similarly to hatched and survived chicks of the other two groups, chicks of group C showed disturbed growth (uneven development). The organs showed lesions of productive nature, mainly accompanied by infiltration with mononuclear cells. As compared to those of the controls, lymphocytes separated on the 20th day after hatching showed expressed rosette-formation indicative of antigen (reovirus) recognition.

The present results agree with our earlier observations (Glávits et al., 1983) that the type of cellular reactions and immune response of the chicken embryo and the day-old chick to a viral antigen, as well as the nature of the developing pathological lesions depend on the developmental status of the lymphatic and myeloid systems at the time of infection. In an earlier infection experiment using lentogenic Newcastle disease virus (Glávits et al., 1983), pathological lesions occurred mostly in the wall of the respiratory tract and blood vessels and in the lymphoid tissues of chicken embryos and day-old chicks. Avian reovirus induced changes in the liver, pancreas, glandular stomach wall and myocardium in all experimental groups. Colonization by lymphocytes of the follicles of the bursa of Fabricius was slower in the infected birds than in the controls.

The results of the present experiments agree with the observations of Menendez et al. (1975) according to which in embryonated eggs infected with a low-titre virus some of the embryos may die and the survivors may hatch with a low vitality (and resistance). The tissue changes observed by us in the hatched and survived chicks were similar to those often found during our diagnostic work in connection with reovirus infection of 2–4 weeks old chicken flocks (Fehérvári et al., 1984).

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PATHOLOGICAL STUDIES IN CHICKEN EMBRYOS AND DAY-OLD CHICKS EXPERIMENTALLY INFECTED WITH *SALMONELLA TYPHIMURIUM* AND *STAPHYLOCOCCUS AUREUS*

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Twenty and 10 SPF chicken embryos were infected on the 6th day (group A) and on the 14th day (group B) of incubation, respectively, with *Salmonella typhimurium* and the same numbers of embryos with *Staphylococcus aureus*. Twenty day-old chicks were infected orally with the same *Salmonella*, and another 20 with the *Staphylococcus*, strain (group C). On each day after inoculation, one bird from each group was examined by morphological and microbiological methods.

The bacteria, applied in low germ counts (10^3) on the outer surface of the shell membrane, penetrated into the egg and damaged primarily the chorioallantoic membrane (CAM), thus resulting in 10–40% embryonic mortality. The *Salmonella* produced no, while the *Staphylococcus* produced occasional, tissue changes in the embryonic organs. As opposed to certain viral infections studied earlier, no morphological signs indicative of cellular or humoral immune response were seen.

Day-old chicks infected per os with *Salmonella typhimurium* developed acute septicaemia, subacute pneumonia, and those infected with *Staphylococcus aureus* developed arthritis and osteomyelitis. Birds succumbed in the acute stage showed an atrophy of the lymphoid organs (bursa of Fabricius, thymus) characterized by depletion of lymphocytes and necrosis, while the lymphoid organs of chicks bled in the subacute stage developed hyperplastic changes.

Keywords. Experimental infection, immune system, chicken embryo, day-old chick, pathology.

In a previous paper we described the embryonic and postembryonic development of the lymphoid and myeloid system of the chicken (Glávits et al., 1981). Furthermore, we have reported on the pathological and immunomorphological changes induced in chicken embryos and day-old chicks by lentogenic Newcastle disease virus (NDV) and avian reovirus. We found that both the resistance of the organism to these pathogenic agents and the type of the lesions produced depended on the developmental status of the lymphatic and myeloid systems (Glávits et al., 1983*a*, *b*).

In the present paper the results of experimental infection with *Salmonella typhimurium* and *Staphylococcus aureus* performed at different times of incubation and at hatching are reported.

Materials and methods

One hundred dwarf Leghorn chicken embryos and 50 day-old chicks coming from an SPF flock were used. The eggs were incubated in a RAGUS-60-type laboratory incubator.

Experimental infection. Twenty and 10 chicken embryos were inoculated on the 6th day (group A) and 14th day (group B) of incubation, respectively, with 0.1 ml of a diluted suspension of a *Salmonella typhimurium* strain (10^3 germs) isolated from chicken, through the air sac on the shell membrane. The same numbers of chicken embryos were infected at the above times with 0.1 ml of a diluted suspension of *Staphylococcus aureus* in the same manner. Forty embryos served as uninoculated control. Twenty one-day-old chicks were infected per os with similar germ counts of *Salmonella typhimurium*, another 20 with *Staphylococcus aureus* (group C). Ten day-old chicks were left as control. One viable embryo, the embryos found dead at candling, and one day-old chick were examined daily from each group.

Histological examination. The entire embryo, the shell membrane, the chorioallantoic membrane (CAM), the yolk sac wall, and the brain, spleen, liver, kidneys, bursa of Fabricius, thymus, lung, bone marrow and one of the knee-joints of the chicks were examined histologically as described earlier (Glávits et al., 1981).

Bacteriological and mycological examinations were made from the yolk sac, the surface of the CAM, the liver of embryos, the liver, heart blood, bone marrow, knee-joint and intestinal tract of chicks, using agar plates containing 5% sheep blood, Drigalski's and Sabouraud's agar media. *Virus isolation* was attempted from the allantoic fluid and from the spleen and lungs of chicks in primary chicken kidney cell cultures.

Results

Infection with Salmonella typhimurium

The mortality of chicken embryos (groups A and B) and day-old chicks (group C) infected with *Salmonella typhimurium* is shown in Fig. 1. At *autopsy* of group A and group B embryos, the succumbed embryos showed more expressed, while the killed ones less pronounced, turbidity of the allantoic and amnionic fluids, hyperaemia, oedema and occasionally diffuse superficial necrosis of the CAM. The embryonic organs showed only hyperaemia of varying degree. The body length (growth) of embryos infected either on the 6th or 14th day of incubation showed no difference from that of control embryos of the same age.

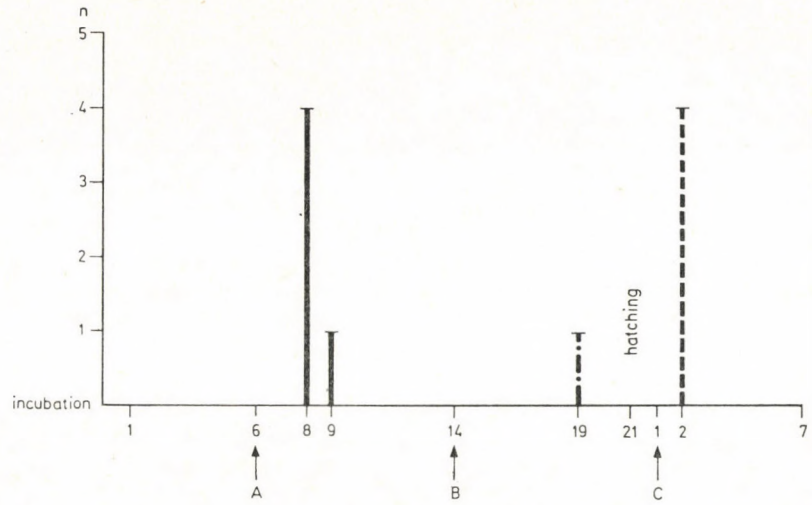

Fig. 1. **Mortality of chicken embryos and day-old chicks infected with *Salmonella typhimurium*. Solid line: Group A, infected on the 6th day of incubation; dotted line: Group B, infected on the 14th day of incubation; broken line: Group C, infected on the 1st day after hatching**

Embryos that had survived up to the time of hatching broke open the egg-shell, but failed to hatch out, as opposed to the controls. No pathological lesions were seen at the autopsy of these embryos.

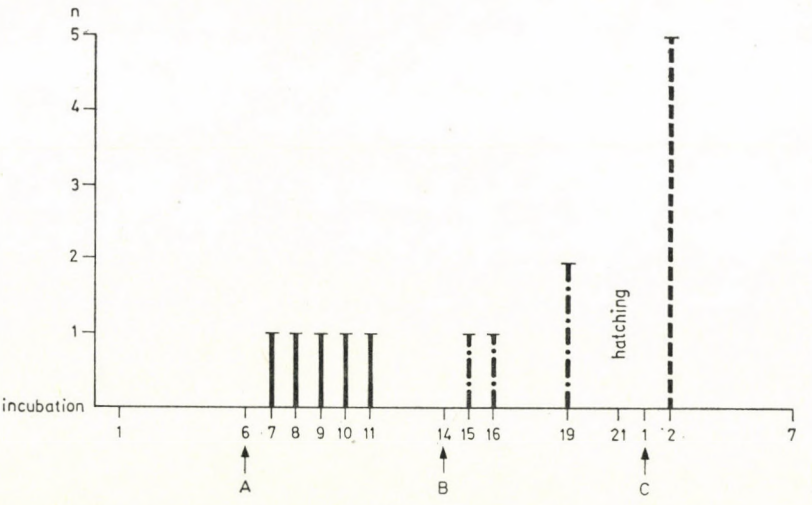

Fig. 2. **Mortality of chicken embryos and day-old chicks infected with *Staphylococcus aureus*. Solid line: Group A, infected on the 6th day of incubation; dotted line: Group B, infected on the 14th day of incubation; broken line: Group C, infected on the 1st day after hatching**

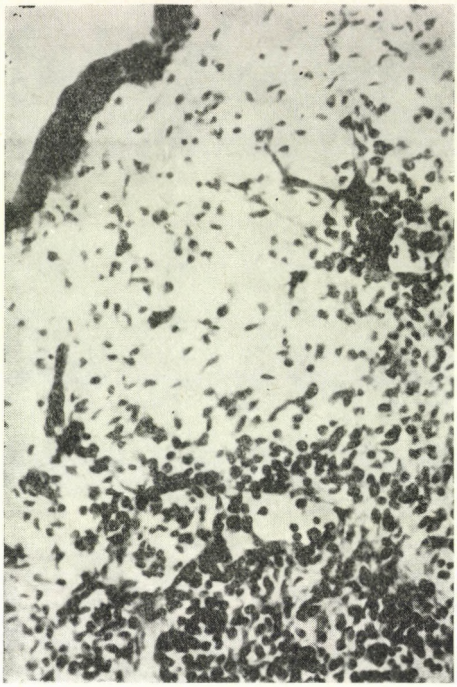

Fig. 3. Dilated vessels surrounded by infiltrating inflammatory cells in the CAM mesenchyma. Group A embryo infected with *Salmonella typhimurium*. Haematoxylin–eosin (H-E.), × appr. 63

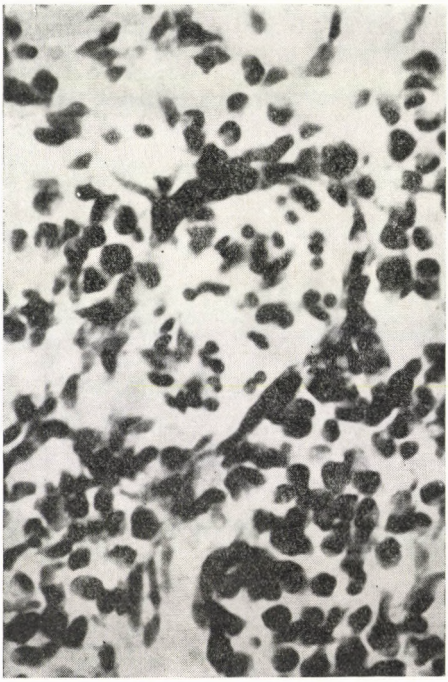

Fig. 4. Detail of Fig. 3. Mainly granulocytes are seen in the lumen of the blood vessel and around it. H-E., × appr. 160

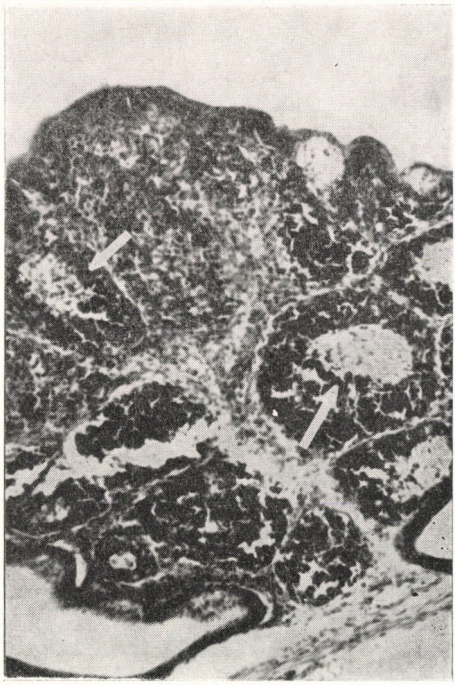

Fig. 5. Depletion of lymphocytes (arrow) in the medullary substance of follicles of the bursa of Fabricius. Group C chick infected with *Salmonella typhimurium*. H-E., × appr. 160

Histological examination of group A and group B embryos revealed high numbers of leucocytes, primarily heterophil granulocytes, in the lumen of dilated blood vessels of the CAM mesenchyma; cells often extravasated (Figs 3 and 4). The bacteria were present in masses on the surface of the CAM epithelium and occasionally also in the mesenchyma. In embryos succumbed to infection, the epithelial layer of the CAM was often necrotic and covered by fibrin. The shell membrane and the yolk sac wall were intact. Signs indicative of normal haemocytopoiesis (haemocytoblasts, erythroid and myeloid "blast" cells) were seen along the blood vessels of the latter.

There were no pathological lesions in the organs of the succumbed and killed embryos.

In group C, the liver and spleen of chicks died of infection showed hyperaemia, activation of RHS cells, and necrotic-inflammatory foci accompanied by the proliferation of reticulocytes. Depletion of lymphocytes was observed in the medullary substance of the bursal follicles (Fig. 5) and in the cortex of lobes of the thymus.

The lymphoid organs of killed group C chicks were free from the above-mentioned changes. The bursa of Fabricius showed follicular hyperplasia. In addition, in three chicks of this group the liver and the spleen showed lesions simi-

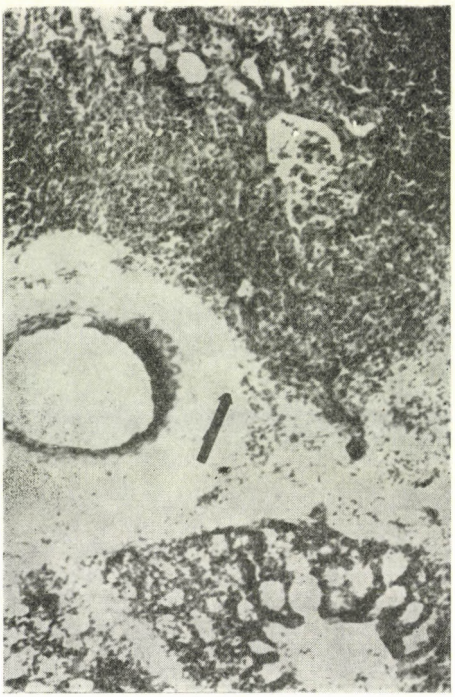

Fig. 6. Focal productive pneumonia characterized by the presence of mononuclear cells (arrow). Group C chick infected with *Salmonella typhimurium*. H-E., × appr. 63

lar to, but milder than, those described above. In the lungs of four chicks there were circumscribed infiltrations by mononuclear cells, extending both to the lumen of parabronchi and to the wall of air and blood capillaries (Fig. 6). The control chicks were free from lesions.

In groups A and B, *Salmonella typhimurium* was recovered from the surface of the CAM of both the succumbed and the killed embryos in rich, carpet-like cultures. The yolk sacs yielded less abundant cultures. The liver of succumbed embryos yielded a few, while that of killed ones only one or two bacterial colonies on culturing. The organism was recovered in rich cultures from the liver, the heart blood, the bone marrow and the intestinal tract of group C chicks died of infection, but from the same organs of killed chicks only a few colonies grew out, and only up to the 6th day post-infection (PI). *Salmonella* organisms were not recovered from the same organs of control chicks. The *mycological* and *virological tests* gave negative results in all groups.

Infection with Staphylococcus aureus

The mortality of chicken embryos (groups A and B) and day-old chicks (group C) infected with *Staphylococcus aureus* is shown in Fig. 2. At autopsy of

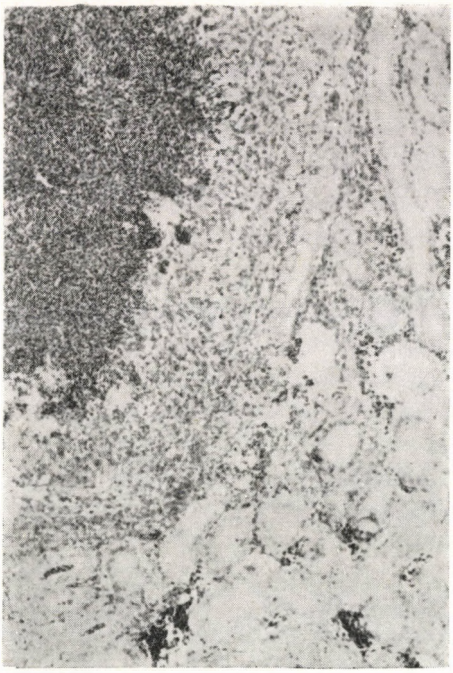

Fig. 7. Demarcated necrotic-inflammatory focus in the kidney of a Group A embryo infected with *Staphylococcus aureus*. H-E., × appr. 63

group A and B embryos, the allantoic and amnionic fluids of embryos and the embryonic membranes showed lesions of similar nature and degree as those found in embryos infected with *Salmonella typhimurium*. The infected embryos did not differ in body length from the controls of the same age.

Embryos surviving up to the time of hatching resembled those of the salmonella-infected group. At autopsy of the survivors no pathological lesions were seen.

According to histological examination of group A and B embryos, inflammation of the CAM was frequently accompanied by necrosis of some epithelial cells from the beginning on. Later on, necrotic lesions spread to the mesenchyma which contained large masses of bacteria. The shell membrane and the yolk sac wall were intact, and the latter showed normal haemocytopoiesis.

In some of the killed embryos the following changes were found. On the 10th day PI the kidneys of a group A embryo (Fig. 7) showed suppurative--necrotic inflammation demarcated by angiofibroblast tissue. Examination of a group B embryo on the 4th day PI revealed an acute, diffuse, serous inflammation accompanied by development of necrotic foci in the liver (Fig. 8) and focal, heterophil granulocytic interstitial inflammation in the kidney at the same time.

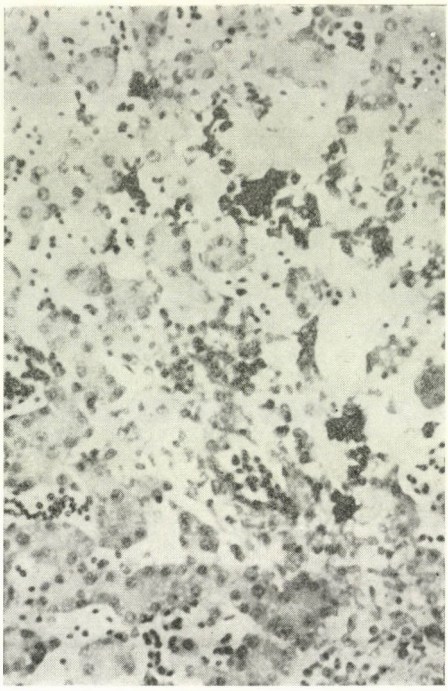

Fig. 8. Serous hepatitis accompanied by circumscribed necrosis of the parenchyma. Group B embryo infected with *Staphylococcus aureus*. H-E., × appr. 160

In group C, the liver of chicks succumbed to infection showed diffuse serous inflammation and contained bacterial emboli, while their spleen showed inflammation accompanied by proliferation of reticulocytes of the red pulp. In the bursa of Fabricius lymphocyte depletion accompanied by necrobiosis (karyorrhexis) of smaller groups of follicular lymphocytes (Fig. 9) was seen, and the cortex of thymic lobules showed depletion of lymphocytes. No pathological lesions were seen in the above organs of killed chicks. Their bursa of Fabricius had follicular hyperplasia. Of the chicks killed on the 6th or 7th day PI, 3 had purulent, fibrinous, necrotic inflammation in the knee-joint (Fig. 10) and focal, purulent osteomyelitis in the bone marrow. These changes were absent from control birds.

In both the died and the killed embryos of groups A and B *Staphylococcus aureus* grew out in rich, carpet-like cultures from the surface of the CAM. However, lesser growth was obtained from the yolk sac, even less colonies grew out from the liver of embryos succumbed to infection, while the liver of only five killed embryos yielded, and even these only 1–2, bacterial colonies. Abundant bacterial growth was obtained in cultures of liver, heart blood, bone marrow, and intestinal tract of group C chicks succumbed to infection. From the blood and organs of killed chicks only one or two colonies grew out occasionally; how-

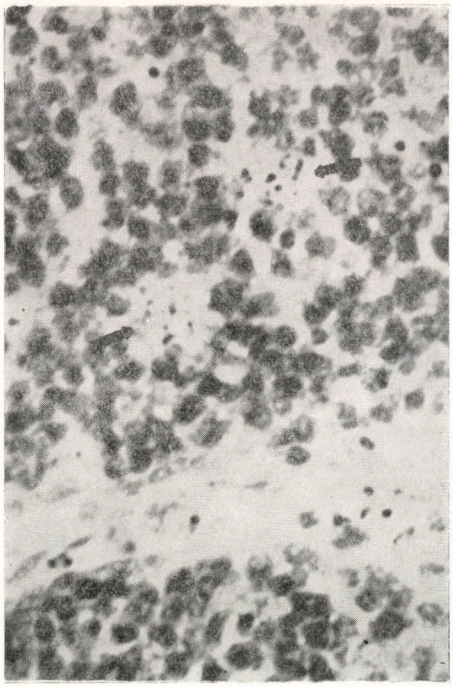

Fig. 9. Necrotic zones (arrow) in the follicles of the bursa of Fabricius. Group C chick infected with *Staphylococcus aureus*. H-E., × appr. 160

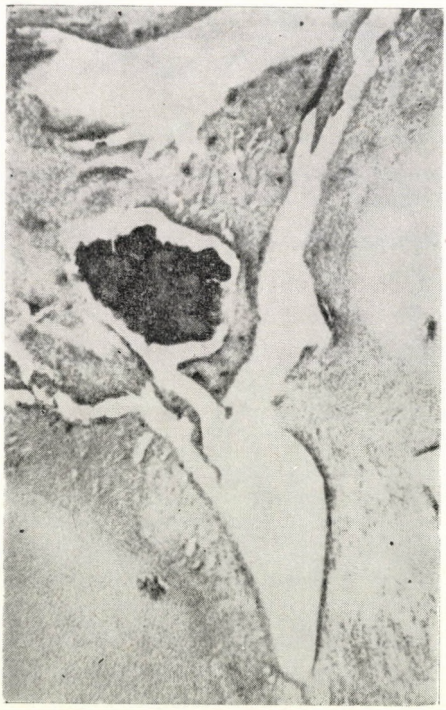

Fig. 10. Purulent necrotic arthritis. Group C chick infected with *Staphylococcus aureus*. H-E., × appr. 25

ever, from the joint cavity and bone marrow of chicks affected with arthritis and osteomyelitis large numbers of *Staphylococcus aureus* colonies developed. The *mycological* and *virological* examinations gave negative results in all groups.

Discussion

Our investigations have revealed that both *Salmonella typhimurium* and *Staphylococcus aureus* applied in low counts (10^3 germs) through the calcic eggshell onto the outer surface of the intact shell membrane penetrated into the egg and damaged primarily the CAM, causing its inflammation. This resulted in death of about 10–40% of the embryos. *Salmonella* organisms produced no, while staphylococci caused only occasional, tissue changes in the embryonic organs. No morphological signs indicative of cellular or humoral immune response were seen. The bacterium could be reisolated in rich cultures from the CAM but only few colonies grew from the organs. The growth rate of infected embryos was not lesser than that of the controls, and most of the embryos survived up to the time of hatching.

The pathogenesis of various viral infections of the embryo strikingly differs from that found in the present experiment. Avian encephalomyelitis virus, infectious bronchitis virus (Casorso and Jungherr, 1959), avian reovirus (Menendez et al., 1975; Glávits et al., 1984) and lentogenic Newcastle disease virus (Glávits et al., 1983*a*, *b)* produce lesions primarily in embryonic organs, which lesions may lead to death, retarded growth or malformation of embryos.

Our earlier investigations have revealed that lentogenic Newcastle disease virus produced characteristic lesions primarily in the respiratory tract and lymphoid organs, while avian reovirus in the liver, heart, pancreas and glandular stomach wall. In the first half of incubation these viruses caused mainly exudative tissue changes in the above organs of embryos, resulting in high mortality. On the other hand, no mortality occurred in the second half of incubation, and the organs contained primarily productive changes. By the end of incubation, signs indicative of cellular and humoral immune response had also appeared (Benedict, 1977; Fehér, 1981; Firth, 1977; Glávits et al., 1983*a*, *b;* Glick, 1979; Tizard, 1977). In experimental infection with *Salmonella typhimurium* and *Staphylococcus aureus* no morphological signs indicating cellular or humoral immune response were seen, presumably due to the different pathogenesis of these infections. In our opinion, this finding can be explained by the fact that these bacteria multiplied mainly in the embryonic membranes and fluids, and thus exerted less effect directly on the lymphoid organs of the developing embryo than the above viral agents.

In chicks infected when day-old, the disease appeared in the form of acute septicaemia or subacute pneumonia, or arthritis and osteomyelitis. We consider

important the observation that in birds died in the acute stage of the disease atrophy of the lymphoid organs (necrobiosis and depletion of lymphocytes), while in birds killed in the subacute stage hyperplastic changes were seen.

Taking into consideration also the conclusions drawn from earlier experiments (Glávits et al., 1983*a*, *b*) it seems that the outcome of infections occurring during incubation depends on the type, virulence and dose of the pathogenic agent and on the time of its penetration into the embryo. These factors determine the pathogenesis of the disease, the morbidity and mortality of embryos and the degree of their active resistance to the pathogenic agent in a given period.

In agreement with observations made in the field, our experiments have shown that the infections occurring during incubation are not always fatal. Embryos may survive, hatch, and lesions produced by the given agent may already be present at hatching, and the birds may become carriers of the pathogenic agent.

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RAPID ANALYTICAL METHOD FOR THE QUANTITATIVE DETERMINATION OF TRICHOTHECENE TOXINS IN FOOD AND FEEDS

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The trichothecene toxins occurring in the purified extracts of food and feed samples can be transformed to corresponding free alcohols by treatment with transesterifying reagents. The transformation of the commonly occurring trichothecene compounds results in three free alcohols, viz. T-2 tetraol toxin, scirpentriol and nivalenol. Deoxynivalenol does not change during this treatment.

SE 52 stationary phase was found to be the most suitable for the separation of mycotoxin-containing samples. Using high-performance thin-layer chromatography (HPTLC) for the analysis of trichothecenols, the R_f values are between 0.05 and 0.1 (benzene–acetone 12 : 7 v/v). By the use of a capillary gas chromatographic method (SE 52 stationary phase) the retention indices of trichothecenols range from 2200 to 2500. In comparative determinations between our former and this new method, the differences in results were less than $\pm 10\%$.

The recommended transesterification method using both HPTLC and gas chromatography is simpler and more reliable than our former method and needs no special standards (e.g. neosolaniol).

Keywords. Trichothecene, mycotoxin, quantitative determination, transesterification, high-performance thin-layer chromatography, gas chromatography.

The 12, 13-epoxy-trichothecenes are chemically related toxic metabolites produced by various species of *Fusarium*, *Cephalosporium*, *Myrothecium* and *Stachybotrys* (Chi et al., 1978).

These fungi are widely distributed in nature and have been isolated from many varieties of crops used for food and feed.

Up to now, more than 40 kinds of trichothecenes have been isolated from the above fungal species (Ohta et al., 1977).

Many of the naturally occurring trichothecenes have been reported as being very toxic to all kinds of animals (Vesonder et al., 1978; Mirocha et al., 1976, 1979).

In practical circumstances the feedstuffs may be contaminated by many mold species. Most of the *Fusarium* species produce several trichothecene mycotoxins at the same time (Table I). Consequently, various toxins may be present in feedstuffs (Table II). The extension of analyses to each trichothecene toxin is a very elaborate work especially by thin-layer chromatography (TLC) methods, for separate working processes must be used for the analysis of the various toxins to achieve sufficiently low detection limits.

To estimate the toxic effect of a feedstuff it is advisable to take into consideration each toxic component. Our aim was to develop a simple and time-saving method to satisfy the above requirements.

Table I

Toxins produced by different *Fusarium* species

Species	Toxins produced
F. nivale	fusarenon X, nivalenol
F. poae	neosolaniol, T-2, HT-2, diacetoxyscirpenol (DAS)
F. sporotrichioides	neosolaniol, T-2, HT-2, DAS
F. roseum	neosolaniol, T-2, HT-2, deoxynivalenol
F. equiseti	neosolaniol, T-2, DAS
F. graminearum	neosolaniol, T-2, HT-2, DAS
F. oxysporum	fusarenon X, T-2, HT-2, DAS
F. solani	neosolaniol, T-2, HT-2, DAS

Table II

Trichothecene toxins in 20 feedstuff samples examined by us earlier

No. of sample	T-2 toxin	HT-2 toxin	DAS	Deoxy-nivalenol	Acetyl T-2 toxin
1	+		+		
2	+		+		
3	+			+	
4	+			+	
5	+			+	
6			+		
7			+		
8			+		
9	+				
10	+				
11	+	+			
12	+	+		+	
13	+		+		
14	+	+			+
15			+		
16			+	+	
17	+	+			+
18			+		
19			+		
20	+				

Materials and methods

Apparatus

a) Vacuum rotary apparatus (Büchi, Flawil, Switzerland);
b) Block thermostat (Pierce Chemical Co., Rockford, Ill., USA);
c) Gas chromatograph Packard Model 427 equipped with FID detector and HP 3390 integrator;
d) Screw-cap vials (Pierce Chemical Co., Rockford, Ill., USA);
e) Glass capillary column, wetted with SE 52 stationary phase, according to the method of Grob et al. (1978).

Reagents and solvents

a) All solvents were of commercial grade, destilled before use (Reanal, Hungary);
b) Mycotoxin standards: T-2 toxin and diacetoxyscirpenol (DAS) were obtained from Supelco; MAS, HT-2 toxin and neosolaniol were kindly supplied by Dr. M. Palyusik; scirpentriol and T-2 tetraol were produced by us;
c) BSTFA (Pierce Chemical Co., Rockford, Ill., USA);
d) Sodium methylate (NaOMet) was prepared in our laboratory (11.5 g sodium + 1000 ml methanol);
e) Silica gel 60 (0.200–0.063 mm) for column chromatography, silica gel 60 plates (0.2 mm thin) for high-performance thin-layer chromatography (HPTLC) (Merck, Darmstadt, FRG).

Extraction and purification

Twenty g of finely ground sample were weighed in a 500 ml Erlenmeyer flask. The sample was extracted with 200 ml of ethyl acetate, then with 200 ml of methanol–water mixture (6 : 4 v/v) on oscillating shaker in two steps. After filtration the two extracts were combined and evaporated on rotary evaporator.

The residue was dissolved in 2 ml of benzene–acetone (2 : 1 v/v) and poured on 10 × 1 cm silica gel column. Subsequently it was washed with 30 ml benzene for defattening. The mycotoxins were eluted with 20 ml benzene–acetone (1 : 1 v/v). The eluate was divided into two portions for gas-liquid chromatography (GLC) and TLC and evaporated to dryness.

Transesterification and derivatization

One portion was dissolved in 10 ml of 0.5 N NaOMet. It was kept at room temperature for 30 min. Six ml of 1 N HCl were added in methanol, mixed, then evaporated to dryness. The residue was dissolved in 1 ml acetone. Five

hundred μl of acetone solution was transferred into a screw-cap vial and the sample was evaporated in N_2 atmosphere. One hundred ml of BSTFA reagent was added, the vial was closed tight and heated for 15 min at 60 °C. The mixture was cooled and injected into the gas chromatograph.

Gas chromatography

Ten m long, 0.30 mm i.d. wetted by SE 52 stationary phase glass capillary column was used at a column temperature of 180–260 °C, 4 °C/min (Grob et al., 1978).

Thin-layer chromatography

Benzene–acetone mixture (12 : 7 v/v) was used for developing. The developed TLC plate was sprayed with 1% 4-(p-nitrobenzyl)-pyridine, heated at 150 °C for 30 min and sprayed with 3% tetraethylene-pentamine solution.The spots of toxins were of blue or lilac blue colour (Takitani et al., 1979). The R_f values of the examined toxins are shown in Table III.

Table III

TLC data of trichothecene mycotoxins

Mycotoxin	R_f value	Detection limit μg per spot
T-2 toxin	0.53	0.1
HT-2 toxin	0.33	0.05
Neosolaniol	0.28	0.1
T-2 tetraol toxin	0.06	0.05
Diacetoxyscirpenol	0.50	0.2
Monoacetoxyscirpenol (MAS)	0.38	0.1
Scirpentriol	0.06	0.1

The developing solvent was benzene—acetone 12 : 7 v/v.

Results and discussion

The results obtained by the analysis of 6 naturally contaminated samples are summarized in Tables IV and V.

The transmethylation reactions were accomplished nearly without loss (Tables IV and V). The new method is suitable for determination of the tricho-

thecene toxin content of samples, and the reliability of the simultaneous determination method evolved earlier in our laboratory (Bata et al., 1983) is enhanced by it. Using special stationary phase, by measuring of 2 peaks, the presence of 7 fusariotoxins can be followed.

Table IV

GLC results of 6 naturally contaminated samples before transesterification

Samples	T-2 toxin	HT-2 toxin	Neosolaniol	T-2 tetraol	DAS	MAS	Scir-pentriol
Maize	0.17	0.05	0.00	0	0.85	0.00	0
Oat	0.72	0.10	0.00	0	0.00	0.00	0
Rye	1.97	0.07	0.85	0	0.00	0.00	0
Mixed feed I	1.18	0.07	0.57	0	0.00	0.00	0
Mixed feed II	1.75	0.27	0.00	0	0.50	0.14	0
Mixed feed III	2.40	0.28	0.15	0	0.75	0.00	0

Table V

Results of the same samples as in Table IV after transesterification

Samples	T-2 toxin	HT-2 toxin	Neosolaniol	T-2 tetraol	DAS	MAS	Scirpentriol
Maize	0	0	0	0.20	0	0	0.80
Oat	0	0	0	0.85	0	0	0
Rye	0	0	0	3.15	0	0	0
Mixed feed I	0	0	0	1.82	0	0	0
Mixed feed II	0	0	0	2.12	0	0	0.76
Mixed feed III	0	0	0	3.00	0	0	0.70

The direct analysis of samples by HPTLC and the reinvestigation after transesterification give new possibilities in the confirmation. The spots corresponding to the individual trichothecene compounds disappear and a new spot appears after transesterification. In our opinion, it is satisfying to determine the end products of transesterification processes for preliminary testing of fusariotoxins. The quantity of T-2 tetraol and scirpentriol is the sum of several trichothecene compounds. In addition to these two toxins, nivalenol may also be formed in smaller amounts. Deoxynivalenol, if present in the sample, will not change during transesterification.

By the increase of the ratio of acetone in the benzene–acetone mixture used for HPTLC, the R_f values of T-2 tetraol and scirpentriol increase, so these

trichothecenols can be determined separately from the contaminating compounds. By the earlier TLC methods the determination of T-2 toxin and DAS was very difficult, sometimes even impossible, due to the interfering effect of contaminating compounds.

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OCCURRENCE OF MYCOTOXINS IN FEEDS, ANIMAL ORGANS AND SECRETIONS

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Results of the mycotoxin analyses performed in the Central Veterinary Institute (Budapest) in years 1977 through 1982 are reported. Of 7345 analyses 930 (12.7%) were positive. Most of the samples were taken from diseased or dead animals. The author describes the considerations determining the direction of examination, i.e. that which are the mycotoxins a sample should be tested for. Routine analysis comprised 11 mycotoxins, using internationally accepted thin-layer chromatographic methods and appropriate confirmation procedures.

The data collected during a one-year period are analysed in detail. These provide information for the practice on the fungal flora and mycotoxin content of feeds and on the clinical symptoms and pathological lesions produced.

Keywords. Occurrence, mycotoxin, feed, organ, secretion.

In the 'sixties of this century it was discovered that fungal contamination of feeds and the consequent mycotoxicoses are of decisive importance in animal production.

Microbiological examination of feeds, particularly determination of their fungal flora, has become widespread in Hungary since that time. In the Central Veterinary Institute it was introduced immediately; the chemical analysis of mycotoxins was started in the 'seventies. Most of the samples sent to this Institute were taken from diseased farm animals or were post-mortem specimens.

In previous papers I have summarized results of the mycological examination of several thousands of feed samples, together with results on the toxin-producing ability of 100 *Aspergillus*, 100 *Fusarium* and 100 *Penicillium* strains (Sellyey-Sándor, 1978, 1981).

Data obtained during 6 years for 11 routinely analysed and 5 occasionally examined mycotoxins are reported in the present paper.

Mycotoxin analyses were performed if (i) the veterinarian called attention to clinical symptoms or pathological lesions indicative of mycotoxicosis, and if (ii) the feed contained toxin-producing fungi in large numbers or in monoflora.

For a year chosen arbitrarily, all the positive results available are presented in detail to demonstrate relationships between the mycological status and the mycotoxin content of the feed on the one hand and the observed clinical symptoms and pathological lesions on the other.

Methods

The determination of the following mycotoxins was performed: zearalenone, T-2 toxin, diacetoxyscirpenol, deoxynivalenol, citrinin, ochratoxin A, patulin, rubratoxin B, aflatoxin B_1, G_1, B_2, G_2, stachybotryotoxins (satratoxins G and H), sterigmatocystin. The toxins were extracted from the samples, appropriately purified, and determined by thin-layer chromatography using UV detection and/or spray reagents, followed by different confirmation methods.

The analysis of samples for alpha- and beta-zearalenol, diacetylnivalenol, HT-2 toxin and T-2 tetraol was performed by gas chromatography (GLC).

For methodical details, see the papers listed under References.

Results and discussion

The results of the mycotoxin analyses performed in the 6-year period between 1977 and 1982 are shown in Tables I, II and III.

Table IV presents in detail the positive results obtained in a one-year period; the type of feed, the number of cells per gram of *Aspergillus*, *Penicillium*

Table I

Distribution of the positive results

Sample	Samples zearalenone					
	1977	1978	1979	1980	1981	1982
Maize	3	5	2	—	11	—
Wheat	2	1	2	1	6	—
Barley	—	—	—	—	—	—
Oat	—	—	—	—	—	—
Soybean groats	—	—	—	—	—	—
Sunflower	—	—	—	—	—	—
Sugar-beet slices	—	—	—	—	—	—
Alfalfa	—	—	—	—	—	—
Hay, straw	—	5	—	—	—	1
Mixed feed	3	7	8	6	11	12
Liver (piglet)	—	—	—	23	—	—
Kidney	—	—	—	—	—	—
Milk	—	—	—	16	—	—
Faeces	—	—	—	—	—	4
No. of positive samples	8	18	12	46	28	17
No. of samples examined	682	203	134	148	215	184
Per cent positive	1.2	8.9	9.0	31.1	13.0	9.2

and *Fusarium* spp. capable of toxin production, case-history data, and the demonstrated mycotoxins are given. All data are shown, since it would have been arbitrary to report only those cases in which the case history had indicated the presence of a certain mycotoxin, later on confirmed by chemical examination. Such an approach would not represent the complexity and many-sidedness of the diagnostic work. It should also be taken into account that the veterinarian submitting the sample for examination makes, or has others make, investigations in other directions, parallel with the laboratory analysis. Thus, he alone is competent to decide upon the aetiological role of the toxin(s) demonstrated in the feed, in the light of all the results obtained.

Sometimes, chemical tests were performed also for toxins apparently unrelated to the case-history, justified by the field veterinarian's request or by results of the mycological examinations. Although such findings do not have diagnostic value in the given case, they may provide useful information as regards further usability of the feed under study.

Table IV contains only the positive results. Of course, many of the "positive" samples gave negative results for other mycotoxins, as well as in other toxicological, bacteriological and virological tests.

by years and feed types

positive for											
T-2 toxin						diacetoxyscirpenol					
1977	1978	1979	1980	1981	1982	1977	1978	1979	1980	1981	1982
3	2	—	2	30	23	1	—	—	—	1	2
2	—	—	6	10	11	—	—	—	—	—	1
—	—	—	2	2	—	—	—	—	—	—	—
—	—	1	1	6	1	—	—	—	—	—	—
—	—	—	—	—	—	—	—	—	—	—	—
—	—	—	1	—	—	—	—	—	1	—	—
—	—	—	—	—	—	—	—	—	—	—	—
—	—	1	1	—	—	—	—	—	—	—	—
3	2	—	4	1	—	—	—	—	—	—	—
11	13	2	91	144	99	—	—	1	3	3	1
—	—	—	—	—	—	—	—	—	—	—	—
—	—	—	—	—	—	—	—	—	—	—	—
—	—	—	—	—	—	—	—	—	—	—	—
—	—	—	—	—	4	—	—	—	—	—	4
19	17	4	108	193	138	1	—	1	4	4	8
843	279	146	387	789	548	18	40	48	102	63	159
2.3	6.1	2.7	28.0	24.5	24.6	5.6	0	2.1	3.9	6.3	5.0

It should be noted that the maize was subjected to drying with warm air before stored. Thus, *Fusarium* spp. sensitive to heat may have been partly or completely destroyed, but their heat-resistant toxins remained active in the feed. Naturally, in such cases the mycological findings are not consistent with the toxin demonstrated.

Nevertheless, in most cases there is an unambiguous relationship between the produced lesions and the demonstrated mycotoxin(s).

Fig. 1 shows the annual summary of the percentage distribution of negative and positive results, and the number of analyses. In the last three years of study, the proportion of positive results increased. In addition to objective, e.g. weather-dependent, conditions, subjective factors may have played a role in this; namely, the samples submitted for analysis were accompanied by a more detailed case-history, allowing us to perform aimed analyses. In addition to this, the employed methods have undergone remarkable development.

Of the 7345 analyses performed during the 6 years of study, 930 (12.7 %) gave positive results.

The results reported in the present paper clearly demonstrate that the routine diagnostic and research work on mycotoxins helps to solve problems arising in veterinary practice and animal production.

Table II

Distribution of the positive results

Sample	Samples					
	deoxynivalenol				citrinin	
	1979	1980	1981	1982	1978	1979
Maize	—	—	2	—	—	—
Wheat	—	—	—	—	—	—
Barley	—	—	—	—	—	1
Oat	—	—	—	—	—	—
Soybean groats	—	—	—	—	—	—
Sunflower	—	—	—	—	—	—
Sugar-beet slices	—	—	—	—	—	—
Alfalfa	—	—	—	—	—	—
Hay, straw	—	—	—	—	1	—
Mixed feed	—	—	4	7	—	—
Liver (piglet)	—	—	—	—	—	—
Kidney	—	—	—	—	—	—
Milk (sow)	—	—	—	—	—	—
Faeces	—	—	—	—	—	—
No. of positive samples	—	—	6	7	1	1
No. of samples examined	2	12	24	50	31	42
Per cent positive	0	0	25	14	3.2	2.4

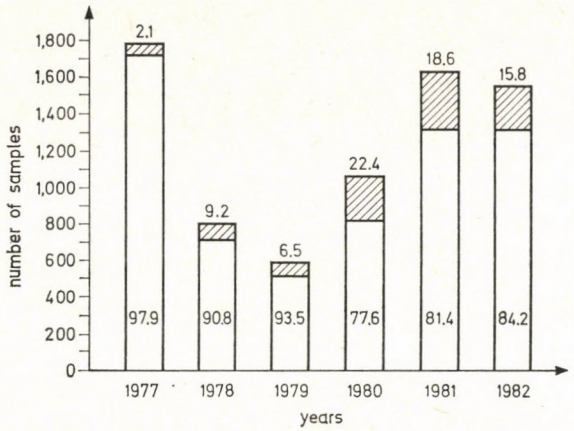

Fig. 1. Percentage distribution of positive and negative results, related to the number of analyses. Striated columns: percentages of positive results; white columns: percentages of negative results

by years and feed types

positive for								
						ochratoxin A		
1980	1981	1982	1977	1978	1979	1980	1981	1982
—	—	6	2	3	—	4	19	23
—	—	1	—	2	—	2	10	8
—	—	—	—	1	1	2	—	2
—	—	—	—	1	1	—	1	1
—	—	—	—	1	1	—	—	—
—	—	—	—	—	—	—	—	—
—	—	—	—	—	1	—	—	—
—	—	—	—	3	—	—	—	—
—	—	—	—	2	—	—	—	—
—	—	2	2	12	8	17	26	16
—	—	—	—	—	—	—	—	—
—	—	—	—	—	—	43	10	10
—	—	—	—	—	—	—	—	—
—	—	—	—	—	—	—	—	—
—	—	9	4	25	12	68	66	60
15	34	85	76	147	122	294	394	317
0	0	10.6	5.3	17	9.8	23.1	16.8	18.9

Table III

Distribution of the positive results

Samples

Sample	patulin					
	1977	1978	1979	1980	1981	1982
Maize	1	—	—	—	—	3
Wheat	—	—	—	—	—	—
Barley	—	—	2	—	—	—
Oat	—	—	—	—	—	—
Soybean groats	—	—	—	—	—	—
Sunflower	—	—	—	—	—	—
Sugar-beet slices	—	—	—	—	—	—
Alfalfa	—	—	—	—	—	—
Hay, straw	1	1	—	—	—	—
Mixed feed	—	2	4	—	—	—
Liver (piglet)	—	—	—	—	—	—
Kidney	—	—	—	—	—	—
Milk (sow)	—	—	—	—	—	—
Faeces	—	—	—	—	—	—
No. of positive samples	2	3	6	—	—	3
No. of samples examined	111	49	62	28	27	78
Per cent positive	1.8	6.1	9.7	0	0	3.8

by years and feed types

positive for							
rubratoxin B					Other mycotoxins, 1977–1982		
1978	1979	1980	1981	1982	No. of samples examined		No. of positive samples
—	—	1	—	—	aflatoxins	107	0
1	1	—	—	—	α-zearalenol	4	4 faeces
—	—	1	—	—	β-zearalenol	4	4 faeces
—	—	—	—	—	diacetylnivalenol	1	1 maize
4	—	—	—	—	HT-2 toxin	1	1 maize
—	—	—	—	—	T-2 tetraol	5	5 faeces, maize
—	—	—	—	—	satratoxin G, H	23	7 straw
—	—	—	—	—	sterigmatocystin	17	2 diet
—	—	—	—	—			
5	—	6	5	1			
—	—	—	—	—			
—	—	—	—	—			
—	—	—	—	—			
—	—	—	—	—			
10	1	8	5	1			
37	22	42	52	57			
27.0	4.5	19.0	9.6	1.8			

Table IV/a

Details of positive results associated with diseases in pigs in a one-year period

Registration number	Examined feed	Case-history	Mould flora, cells per gram	Mycotoxin demonstrated
170	maize	vomiting	96,000 P	zearalenone deoxynivalenol griseofulvin
171	maize	vomiting	5,000 P	zearalenone deoxynivalenol griseofulvin
172	maize	vomiting	440,000 P	zearalenone deoxynivalenol griseofulvin
933	wheat	diseases of the digestive system	400 A 300 P	rubratoxin B ochratoxin A
1066	mixed feed	gastroenteritis	2,400 A	T-2 toxin
1067	mixed feed	gastroenteritis	800 P 300 A	T-2 toxin
1528	mixed feed	deaths among fattened pigs	4,000 P	T-2 toxin
1717	soybean groats	inappetence, haemorrhagic gastroenteritis, hepatitis, occasionally myocardial degeneration, blood-streaked diarrhoea	2,000 A 1,000 P	rubratoxin B
1718	soybean groats	inappetence, haemorrhagic gastroenteritis, hepatitis, occasionally myocardial degeneration, blood-streaked diarrhoea	5,000 P 2,000 A	rubratoxin B
1719	soybean groats	inappetence, haemorrhagic gastroenteritis, hepatitis, occasionally myocardial degeneration, blood-streaked diarrhoea	4,000 P 1,000 A	rubratoxin B
1755	piglet diet	gastroenteritis	96,000 P	ochratoxin A
2439	maize	diarrhoea in sows and piglets	74,000 P	ochratoxin A
2440	maize	diarrhoea in sows and piglets	43,000 P	ochratoxin A
2475	piglet diet	sudden death, gastroenteritis	22,000 P	ochratoxin A
2715	piglet diet	diarrhoea in sows and piglets	3,000 F 3,000 A 12,000 P	T-2 toxin
2718	piglet diet	diarrhoea in sows and piglets	1,000 F 3,000 A 1,000 P	T-2 toxin
3120	pig grower diet	diarrhoea, death	1,000 A 22,000 P	rubratoxin B
3633	wheat for sow diet	inappetence and diarrhoea in 1–2 days old piglets	3,000 P 4,000 A	ochratoxin A
3820	piglet diet	liver and kidney degeneration, intestinal catarrh	2,000 P 2,000 A	patulin

Table IV/a (continued)

Registration number	Examined feed	Case-history	Mould flora, cells per gram	Mycotoxin demonstrated
4112	pig grower diet	death, gastroenteritis, liver degeneration	15,000 A 2,000 P	ochratoxin A
4719	fattening pig diet	death	38,000 P 6,000 A	rubratoxin B
5474	maize for diet	piglet diarrhoea from the 2nd–3rd day after ingestion of the diet	5,000 F 4,000 P 4,000 A	T-2 toxin
5475	wheat	piglet diarrhoea from the 2nd–3rd day after ingestion of the diet	49,000 P	ochratoxin A patulin
5476	barley	piglet diarrhoea from the 2nd–3rd day after ingestion of the diet	75,000 A 25,000 P	ochratoxin A
6134	pig grower diet	diarrhoea, inappetence	5,000 F 5,000 P 2,000 A	T-2 toxin

A = *Aspergillus;* F = *Fusarium;* P = *Penicillium*

Table IV/b

Details of positive results associated with diseases in poultry in a one-year period

Registration number	Examined feed	Case-history	Mould flora, cells per gram	Mycotoxin demonstrated
89	diet	depression and diarrhoea in laying hens	4,000 A 5,000 P	zearalenone, ?
90	diet	depression and diarrhoea in laying hens	600 A	zearalenone, ?
91	diet	depression and diarrhoea in laying hens	100 A 300 P	zearalenone, ?
92	diet	depression and diarrhoea in laying hens	100 P	zearalenone, ?
93	diet	depression and diarrhoea in laying hens	11,000 A 3,000 P	zearalenone, ?
491	starter diet	broiler chick death; prior to death the birds were ataxic, staggered, showed disturbed feathering, the glandular stomach contents were bloody, haemorrhages were seen around the outlet ducts of glands and on the caecal valves, feed consumption was reduced	8,000 P 14,000 A	rubratoxin B
494	starter diet	broiler chick death; prior to death the birds were ataxic, staggered, showed disturbed feathering, the glandular stomach	700 A 1,000 P	rubratoxin B

Table IV/b (continued)

Registration number	Examined feed	Case-history	Mould flora, cells per gram	Mycotoxin demonstrated
		contents were bloody, haemorrhages were seen around the outlet ducts of glands and on the caecal valves, feed consumption was reduced		
1052	layer feed	disturbed feathering, feed refusal, dropped egg production, death	18,000 P	T-2 toxin
1053	layer feed	disturbed feathering, feed refusal, dropped egg production, death	8,000 P	T-2 toxin
1054	layer feed	disturbed feathering, feed refusal, dropped egg production, death	9,000 P 2,000 A	T-2 toxin
1055	layer feed	disturbed feathering, feed refusal, dropped egg production, death	13,000 P 3,000 A	T-2 toxin
2380	diet	loss of appetite, retarded growth, enteritis, liver and kidney degeneration	152,000 P	ochratoxin A
3455	diet	haemorrhages in the caecum and small intestine	4,000 A 21,000 P	zearalenone, ?
4909	starter diet	deaths among broiler turkeys, degeneration of the inner organs	1,000 A	ochratoxin A
4924	diet	enteritis, liver and kidney degeneration	2,000 A 17,000 P	ochratoxin A
5133	diet	deaths among 6 weeks old chicks, intestinal catarrh, kidney degeneration	2,000 F 5,000 P 4,000 A	rubratoxin B
5407	starter diet	deaths among broiler turkeys	1,000 A	ochratoxin A
5641	diet	deaths among 6 weeks old chicks	5,000 F	T-2 toxin
6058	diet	enteritis, liver and kidney degeneration	5,000 P	rubratoxin B
6147	grower diet	enteritis, kidney degeneration, haemorrhages and ulcers in the gizzard	—	rubratoxin B
6213	layer feed	loss of appetite, dropped egg production	4,000 P	T-2 toxin
7128	diet	death of hens, roosters, ducks: liver and kidney degeneration, coronary haemorrhages	27,000 P	ochratoxin A
7371	starter diet	liver and kidney degeneration, inflammation of the small intestine	6,000 F 4,000 A 4,000 P	ochratoxin A

A = *Aspergillus;* F = *Fusarium;* P = *Penicillium*

Table IV/c

Details of positive results associated with diseases in cattle in a one-year period

Registration number	Examined feed	Case-history	Mould flora, cells per gram	Mycotoxin demonstrated
57	mixed feed	death of 2–3 weeks old calves within 12 h, enteritis	46,000 P	ochratoxin A citrinin
369	wheat	abortion of 2.5–8 months old fetuses	1,000 F 3,000 P	zearalenone
464	hay	abortion	10,000 F 100,000 P 10,000 A	zearalenone
1124	soybean groats	liver damage, death	400 P	ochratoxin A
1194	meadow hay	weight loss, inappetence	10,000 F 20,000 A	zearalenone, ?
1455	alfalfa	erosions on the oral and pharyngeal mucosa in 2–6 weeks old calves	60,000 F	zearalenone, T-2 toxin
1457	dairy cow diet	erosions on the oral and pharyngeal mucosa	1,000 F 7,000 P 4,000 A	zearalenone
3616	dairy cow diet	gastroenteritis	16,000 A 38,000 P	ochratoxin A
4533	hay	death of young heifers, pulmonary oedema, haemorrhages on the heart, haemorrhagic enteritis in the small intestine	100,000 A	citrinin
6222	hay	death	100,000 F	T-2 toxin
6250	grower diet	diarrhoea	9,000 P	patulin
6251	grower diet	diarrhoea	79,000 A	T-2 toxin
6460	rye	inappetence, weight loss	2,000 F 6,000 P 6,000 A	T-2 toxin
6461	hay	inappetence, weight loss	25,000 F	T-2 toxin

A = *Aspergillus;* F = *Fusarium;* P = *Penicillium*

Table IV/d

Details of positive results associated with diseases of other animals and of those lacking case-history data, in a one-year period

Registration number	Examined feed	Case-history	Mould flora, cells per gram	Mycotoxin demonstrated
20	mixed feed	fatal cases in sheep; loss of fleece, reddening of the skin	3,000 P	zearalenone, ?
858	maize	unknown	1,000 P 2,000 A	ochratoxin A
1436	soybean groats	unknown	1,000 P 2,000 A	rubratoxin B
1829	hay	unknown	10,000 F 110,000 A	zearalenone
1853	maize	inappetence in sheep	3,000 A 50,000 P	zearalenone, ?
2873	hay	unknown	230,000 A 70,000 P	ochratoxin A patulin
2877	straw	unknown	10,000,000 P	ochratoxin A
3643	alfalfa	unknown	20,000 P 40,000 F	ochratoxin A
3645	alfalfa	unknown	40,000 P 50,000 F	ochratoxin A T-2 toxin, diacetoxyscirpenol
3647	alfalfa	unknown	20,000 F	ochratoxin A
3894	starter diet	high mortality among pheasants	30,000 P	ochratoxin A
4615	diet	neurological symptoms, enteritis, liver and kidney degeneration in pheasants	31,000 P 1,000 A	ochratoxin A
4878	oat	death of 6 months old foals; prior to death, severe diarrhoea, increased water consumption, staggering; one cow that had consumed only oat-grits also died as well as 3 pigs; acute gastroenteritis, liver degeneration	33,000 P	ochratoxin A

A = *Aspergillus;* F = *Fusarium;* P = *Penicillium*

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COMPARISON OF THE STRUCTURE OF *SARCOCYSTIS CUNICULI* OF THE EUROPEAN RABBIT *(ORYCTOLAGUS CUNICULUS)* AND *SARCOCYSTIS LEPORUM* OF THE COTTONTAIL RABBIT *(SYLVILAGUS FLORIDANUS)* BY LIGHT AND ELECTRON MICROSCOPY

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The tissue cysts of *Sarcocystis cuniculi* of the European rabbit *(Oryctolagus cuniculus)* and *Sarcocystis leporum* of the cottontail rabbit *(Sylvilagus floridanus)* have been studied by light and electron microscopy. The micromorphology of the tissue cysts of the two rodent species reveals only minor differences, in cyst size, dimensions and shape of the cyst wall projections and the amount of ground substance. Cross-transmission experiments are needed to determine the relationships between the two *Sarcocystis* species.

Keywords. *Sarcocystis cuniculi*, *Sarcocystis leporum*, cyst, structure, light microscopy, electron microscopy, European rabbit, cottontail rabbit.

In 1883, Balbiani proposed the name Sarcosporidia for the protozoan parasites which were seen by Miescher earlier (1843) in muscles of a mouse. Since Rommel and Heydorn (1972) discovered the life cycle of the Sarcosporidia as a coccidian life cycle alternating between an intermediate host (a prey animal) and a definitive host (a predator), many publications on these protozoan parasites have appeared (Mehlhorn and Heydorn, 1978; Levine and Tadros, 1980; Tadros and Laarman, 1982). As the name implies, *Sarcocystis* is found in muscles of the intermediate host: in reptiles, birds and mostly herbivorous mammals. Within the sarcocysts thousands of merozoites and metrocytes are produced by merogony (endodyogeny). Several studies of rabbit *Sarcocystis* have already been done. For example, in 1977 Fayer and Kradel described the life cycle of *Sarcocystis leporum* parasitizing the muscles of the Eastern cottontail rabbit *(Sylvilagus floridanus)* in the USA. Cats and racoons were reported as final hosts in which gamogony and sporogony occur. Tadros and Laarman (1977) studied *S.*

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cuniculi of the European rabbit *(Oryctolagus cuniculus)* in the Netherlands. Domestic cats were found to be the final hosts.

The present work deals with the comparison of *Sarcocystis leporum* in the Eastern cottontail rabbit *(Sylvilagus floridanus)* and *S. cuniculi* in the European rabbit *(Oryctolagus cuniculus)* by light and electron microscopy. Also, the prevalence of *S. cuniculi* in the area around Bonn, West Germany, is reported.

Materials and methods

Sixty-five European rabbits were collected in the Bonn area including 29 in July–September 1980 and 36 in January–March 1982. Twelve cottontail rabbits were collected in Berrien Springs, Michigan, during the fall of 1980 and the spring of 1981. Samples of skeletal muscles, diaphragm, and tongue of the rabbits were minced and treated with trypsin for the detection of merozoites. Heavily infected tissues were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4, at 4 °C for 4–12 h. After being washed in cacodylate buffer, the material was post-fixed in buffered 1.5% OsO_4 for 4 h, washed in buffer and dehydrated in ascending grades of ethanol. While in 70% ethanol, the material was stained with 1.5% uranyl acetate and phosphotungstic acid. After final dehydration in ethanol and clearing in propylene oxide, the material was embedded in Araldite or in Dow epoxy resin. Sections were cut on a Reichert ultramicrotome, stained with uranyl acetate and lead citrate, and examined in a Zeiss EM 9 S2 electron microscope.

Results

Prevalence of S. cuniculi in the Bonn area

Eleven of 29 (37.9%) *O. cuniculus* collected in July–September 1980 were positive for *S. cuniculi*, but only 1 of 36 (2.8%) rabbits collected in January–March, 1982 were infected. Thus, the prevalence of *S. cuniculi* was 18.5% (12 of 65), while in the American cottontail, the prevalence of *S. leporum* was 66.6% (8 of 12).

Light microscopy

The sarcocysts of *S. cuniculi* measured 1.0–1.5 mm in length and 125–200 μm in diameter. Each sarcocyst was subdivided into typical compartments by septa and contained metrocytes and banana-shaped merozoites (Figs 1 and 2). The striated wall was up to 10 μm thick. The sarcocysts of *S. leporum* were spindle-shaped and measured 1.0–5.0 mm by 100–400 μm.

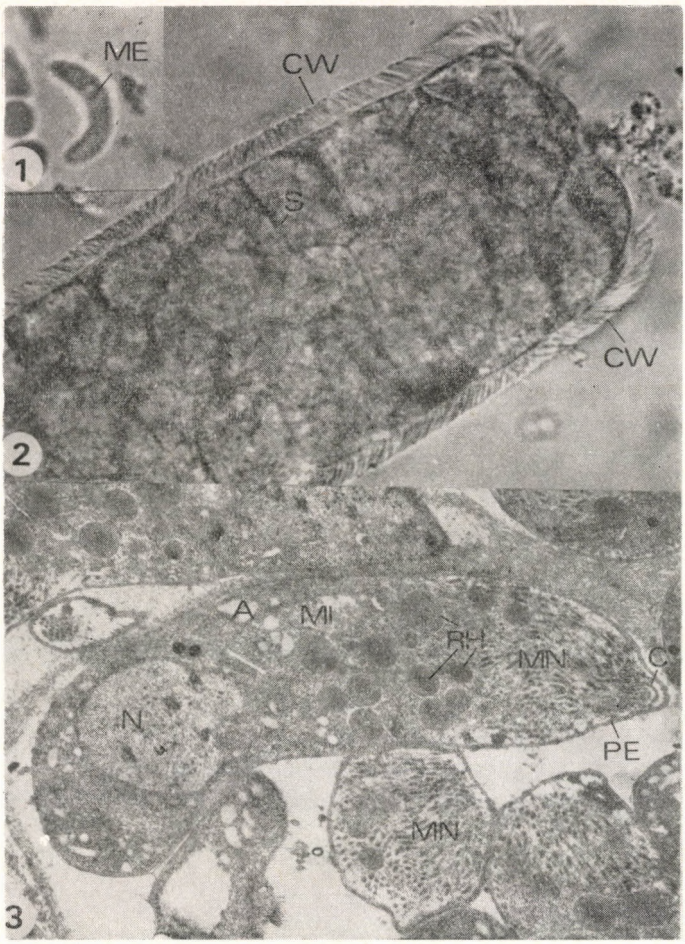

Fig. 1. Sarcocystis cuniculi free merozoite (ME) from the skeletal muscles of the European rabbit. Light micrograph. × 1300

Fig. 2. S. cuniculi. Detached sarcocyst from the skeletal muscle showing the striated cyst wall (CW) and the division into compartments by septa (S). Light micrograph. × 575

Fig. 3. S. cuniculi. Electron micrograph of the merozoite (ME) with the apical complex characteristics; conoid (C), mitochondria (MI), micronemes (MN), pellicle (PE), rhoptries (RH), amylopectin (A) and nucleus (N). × 28,000

Electron microscopy

S. cuniculi

The cysts were bounded by an electron-dense primary cyst wall which bordered the numerous finger-like projections that had appeared as striations by light microscopy (Fig. 4). The projections contained numerous fibrillar elements which originated in the granular ground substance and extended longitudinally to the tips of the projections (Fig. 4). In cross-section the projections

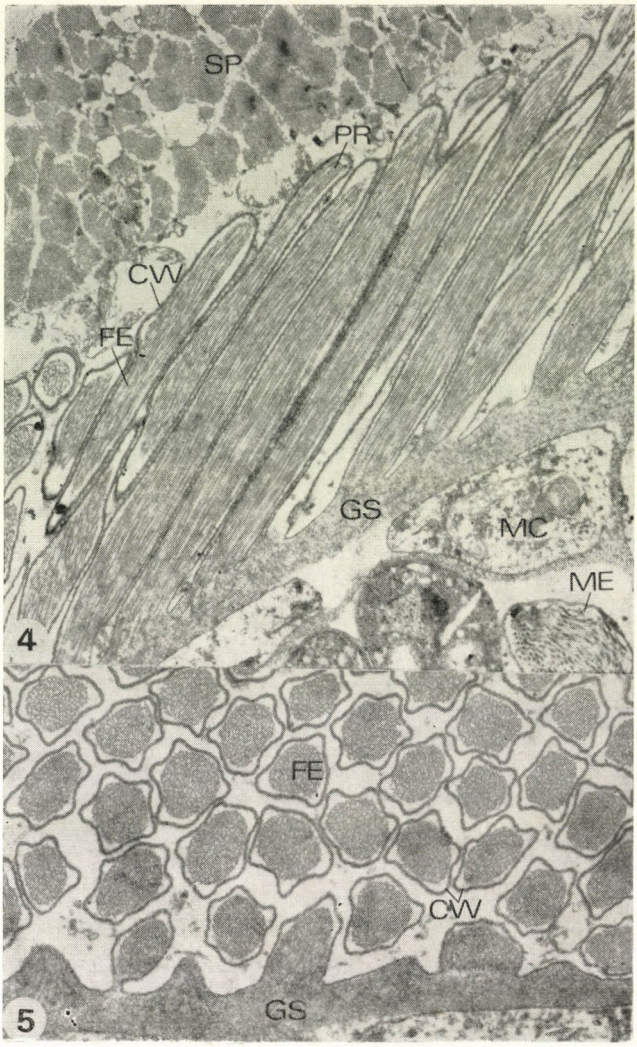

Fig. 4. S. cuniculi. Section through the cyst wall (CW) showing the finger-like protrusions (PR) containing fibrillar elements (FE). × 7300

Fig. 5. S. cuniculi. Cross-section of the cyst wall protrusions with their fibrillar elements (FE). × 12,000

had an irregular shape, with an electron-pale space between the centrally concentrated fibrillar elements and the primary cyst wall (Fig. 5).

In longitudinal sections the primary cyst wall was finely serrated along the projections (Fig. 4); the serrations were not visible in cross-sections (Fig. 5).

The ground substance appeared as a granular band up to 1.5 μm thick just beneath the projections. At various places thin branches from the band extend-

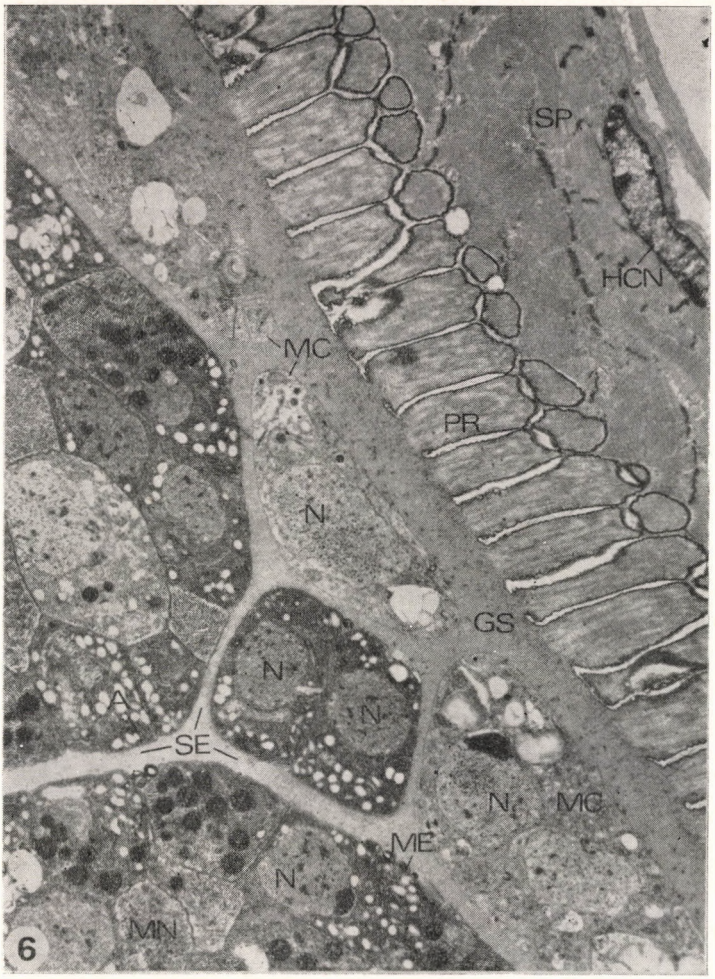

Fig. 6. Sarcocystis leporum in the skeletal muscle of the cottontail rabbit. Electron micrograph of the marginal part of a cyst with prominent protrusions (PR) bordering the sarcoplasm (SP) of the host muscle cell with nucleus (HCN). The cyst ground substance (GS) contains metrocytes (MC) at the periphery, and groups of merozoites (ME) divided by septa (SE). × 6350

ed into the interior, dividing the cyst into compartments in which the metrocytes and merozoites were located.

The metrocytes were few in number in comparison to the merozoites, and were observed at the periphery of the cyst. The metrocytes had an irregular shape, and their electron-pale cytoplasm easily distinguished them from the merozoites (Fig. 4).

The merozoites of *S. cuniculi* measured 10–12 μm × 2–3 μm and had the typical fine structural elements of *Sarcocystis*. Each merozoite was surrounded

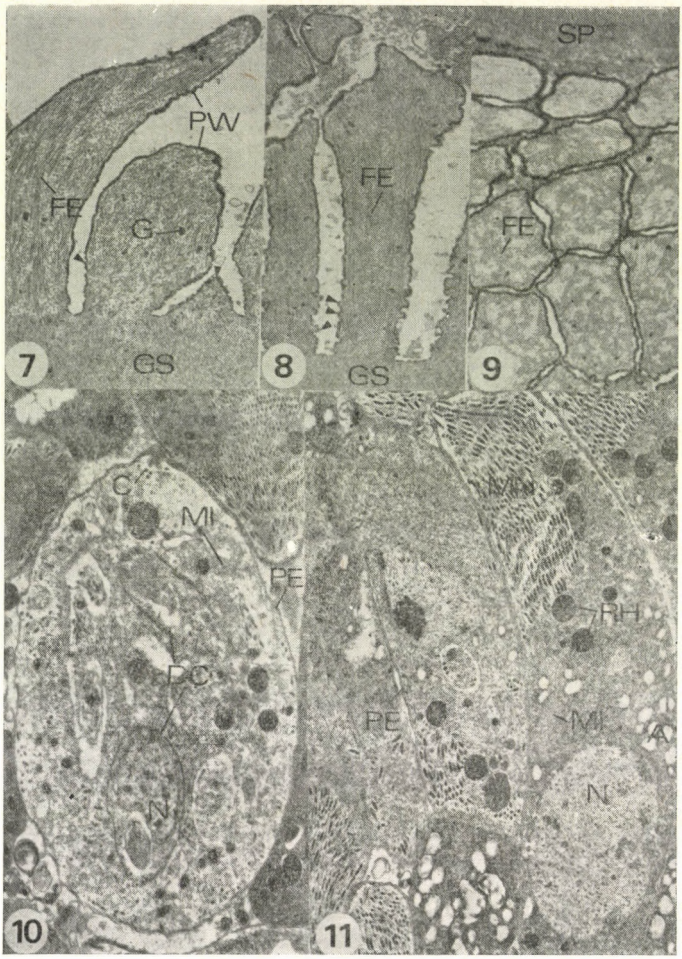

Fig. 7. S. leporum. Longitudinal section through protrusions formed by the primary cyst wall (PW). Fibrillar elements (FE) and granules (G) of different size are visible within the projection. × 12,600

Fig. 8. S. leporum. Longitudinal section through a distally expanded protrusion. Note the folded area of the primary cyst wall at the base of the protrusion (arrows). × 16,000

Fig. 9. S. leporum. Cross-section through protrusions showing fibrillar elements (FE). × 11,700

Fig. 10. S. leporum. Metrocyte with two forming daughter cells (DC), mitochondrion (MI), micronemes (MN), conoid (C) and pellicle (PE). × 11,600

Fig. 11. S. leporum. Longitudinal section through a merozoite with numerous micronemes (MN) and cross sections of rhoptries (RH), mitochondrion (MI), nucleus (N) and polysaccharide granules (A). × 10,000

by a three-membranous pellicle and contained a conoid with a polar ring, numerous micronemes and about 15 bodies considered to be rhoptries in their anterior region (Fig. 3).

S. leporum

The sarcocyts of *S. leporum* were larger than those of *S. cuniculi*, but they revealed similarities in their fine structure to *S. cuniculi*. The cyst wall formed finger-like protrusions, which were mostly thicker and shorter (up to 6 μm long) than the elongated thin protrusions of *S. cuniculi* (Fig. 6). The longitudinally oriented fibrillar elements found within the projections of *S. leporum* occurred in bundles, and they did not extend along the whole length of the projections (Figs 7–9). In addition, the projections contained some scattered osmiophilic particles. The ground substance was nearly identical in appearance with that of *S. cuniculi* but about twice as thick (up to 3 μm); septa originating with the ground substance were also present (Fig. 6).

The metrocytes of *S. leporum* were ovoid in shape, bounded by a three-membranous pellicle, with electron-pale cytoplasm containing a nucleus, micronemes and a conoid (Fig. 10). The merozoites were nearly identical in their fine structure with those of *S. cuniculi;* they were banana-shaped, with a broader posterior part containing the nucleus, and a pointed apical complex (Fig. 11). The merozoites measured 8–13 $\times$ 2–4 μm.

Discussion

Research done on *Sarcocystis* spp. in the last 15 years is so enormous that it is surpassed only by *Plasmodium* and *Toxoplasma* research. Nevertheless, descriptions of new species and experimental studies done with the electron microscope on the sarcocysts and on the sexual stages have continued to enrich our knowledge of this important parasite.

The prevalence of *S. cuniculi* in the Bonn area showed a fluctuating seasonal trend. In the summer of 1980, 37.9% of the examined rabbits *(Oryctolagus cuniculus)* were found to be infected whereas in the winter of 1982 the infection rate dropped to about 2.8%. Collins and Charleston (1979) reported the prevalence of *S. cuniculi* in New Zealand to be at 16%. Munday et al. (1980) studied *S. cuniculi* in *Oryctolagus cuniculus* collected in northern Tasmania and found 6 of 8 (75%) rabbits infected, and Černá et al. (1981) reported a prevalence of 36%. The prevalence of *S. leporum* in cottontail rabbits in Michigan *(Sylvilagus floridanus)* was found to be 66.6%. Other studies of *S. leporum* reported infections of 83.3% (Fayer and Kradel, 1977), and 54.1% (Cosgrove et al., 1982). The reason for the wide range in the prevalence of *Sarcocystis* in rabbits shown by these reports has not been thoroughly studied and is difficult to explain. Possible variables include the surveying of animals with a wide age range (younger rabbits would tend to show lower infection rates), variability in the methods of examination of tissues by different workers, and fluctuation

in the infection rates of the predator animals which disperse the sporocysts of *Sarcocystis*.

In general, the cyst wall of *S. cuniculi* and *S. leporum* is similar to the thick-walled cysts with finger-like projections found in several species of *Sarcocystis* (Mehlhorn and Heydorn, 1978; Entzeroth, 1982). The light and electron microscopic details of the cyst of *S. cuniculi* in the present study are identical to those described in this species earlier (Tadros and Laarman, 1978). Comparisons between *S. cuniculi* and *S. leporum* reveal only minor morphological differences in cyst size, dimensions and shape of the cyst wall projections, and the amount of ground substance. Although the present data show a close similarity of these two species of *Sarcocystis*, cross-transmission studies are needed to determine also their physiological relationship, including host specificity.

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IN VITRO CULTIVATION OF *TRYPANOPLASMA* STRAINS ISOLATED FROM PIKE AND LEECH (PRELIMINARY REPORT)

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A *Trypanoplasma* strain was isolated from the pike *(Esox lucius)* and another from the leech *(Piscicola geometra)*. The isolates were cultivated by serial passages in SNB-9 diphasic blood agar medium at 20 °C. The in vitro development of the strains is reported briefly.

Keywords. *Trypanoplasma*, in vitro cultivation, pike, leech (*Piscicola geometra*).

Trypanosoma and *Trypanoplasma* species, which are haemoflagellates belonging to the Kinetoplastida order, are frequent parasites of both the feral fishes and fishes cultured in pond farms. Although the two genera markedly differ in morphology, they are similar in biology, development and, presumably, pathogenicity.

While *Trypanosoma* spp. parasitizing fishes have been studied extensively from both the morphological and the development-biological point of view, little attention has been paid so far to *Trypanoplasma* spp. The basis of experimental work and research on haemoflagellates is constituted by their isolation and in vitro cultivation. The above-mentioned situation is well reflected by the fact that while research workers have succeeded in cultivating numerous *Trypanosoma* species, only two reliable reports are available as regards the culture of *Trypanoplasma* spp.

Ponselle (1913) succeeded in culturing on an NNN medium modified by him the species *Trypanoplasma varium* Léger isolated from the peripheral blood of *Nemachilus barbatulus*. However, he failed to give data on the duration of culturing and the number of subcultures. Subsequently several research workers have attempted in vitro culturing but, presumably, failed in establishing subcultures from the isolates and gave no data relating to this (Tanabe, 1924; Nowicki, 1940). On a modified NNN medium, Quadri (1962) successfully maintained in continuous culture, through several passages, a *Trypanoplasma* sp. isolated from the peripheral blood of *Salvelinus willoughbii*. Lom (1979) could not maintain in continuous culture the species *T. borelli* isolated from common carp.

All attempts at culturing *Trypanoplasma* spp. were made with flagellates obtained under sterile conditions from the peripheral blood of fish. No isolation and culturing attempts were made from the leech species acting as intermediate hosts.

In the present paper the successful isolation and maintenance in continuous culture of the *Trypanoplasma* strains isolated from peripheral blood of the pike *(Esox lucius)* and from *Piscicola geometra*, a leech collected from common carp, are reported.

The isolates were cultured in an SNB-9 diphasic blood agar medium prepared with 20% inactivated human blood (an outdated batch). Forty μg oxytetracycline was added to the supernatant to prevent bacterial infection. The optimal incubation temperature is 20 °C. Trypanoplasms are more susceptible to high temperature than trypanosomes. This was indicated by the fact that at 25 °C the strains cultured by us failed to divide further, but between 10 °C and 20 °C retained their ability to divide.

The cultures were passaged every 14 days, as at 20 °C the logarithmic phase of growth was reached on the 14th day. A limited growth was observed up to the 18th day. The strain isolated from leech showed considerably better growth and reached a concentration of 5×10^7 specimens/ml, whereas the strain isolated from pike reached a concentration of only 5×10^5/ml, due to its markedly poorer growth.

In the leech, short and wide flagellates were seen together with long and slender ones. The forms isolated from the blood of the pike uniformly corresponded to the typical bloodstream form of *Trypanoplasma*. In culture both strains exhibited wide variation in morphology. Both the forms corresponding to those living in the bloodstream and the long, slender ones occurring in the leech could be found in the subcultures. Transformation of the long, slender forms into rounded, often spherical, stages took place on the 3rd day of culturing. The rounded forms, often having very long flagella, divided intensely. Division was mostly binary, but in some cases multiple. Having completed division, individual flagellates did not separate immediately, but remained attached to one another, forming groups. After multiple fission, groups consisting of numerous flagellates were formed. After the 4th or 5th day the long, slender forms become predominant again, and on the 14th day spherical forms occur only occasionally. During in vitro culturing, in addition to flagellates of regular shape, irregular forms also appear.

Our attempts to infect the common carp and the goldfish with the cultured *Trypanoplasma* strains failed.

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REVERSE RADIOIMMUNOASSAY FOR SEPARATION OF IODOTHYRONINES (RESEARCH NOTES)

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An immunological method for separation of labelled iodothyronines originating from biological fluids is described. By using the method, artefact formation can be avoided and the efficacy of sample processing for serial metabolic clearance rate studies of thyroid hormones can be enhanced.

Keywords. Reverse radioimmunoassay, separation, iodothyronines.

It has been shown recently that a considerable part (30% or more) of the circulating triiodothyronine (T_3) is formed from the thyroxine (T_4) secreted by the thyroid and is the product of peripheral monodeiodination of the T_4. This recognition makes it inevitable to separate iodocompounds from a given plasma sample when kinetic studies of thyroxine metabolism are carried out. Adequate conclusions on metabolic processes cannot be drawn any more from measuring the injected labelled thyroxine after TCA (trichloroacetic acid) precipitation. In order to obtain relevant data on thyroxine metabolism, three important ways of separation have been introduced instead of measuring whole plasma radioactivity or protein-bound iodine (PBI); viz. paper chromatography (Bellabarba et al., 1969), thin-layer chromatography (West et al., 1965) and column chromatography of different kinds (Blasi and DeMasi, 1967; Green, 1972; Tóth, 1978). The two former procedures have the disadvantage of requiring extraction before direct application, the latter, on the other hand, needs large sample quantities. However, the rapid development of the separation techniques has made it possible to prove the possibility of T_4 to T_3 conversion in normal human beings (Sterling et al., 1970), at the same time it has been demonstrated that the separation procedures applied may cause artefact formation. Artefacts may be formed, first of all, through "in vitro" deiodination in the course of chromatography (Fisher and Dussault, 1971; Stanford and Golder, 1975) or while biological materials are extracted by means of different solvent systems (see Fisher and Dussault, 1971; Bellabarba et al., 1969; Volpert et al., 1967).

The application of an appropriate separation procedure is important even in species exhibiting relatively slow thyroxine metabolism, e.g. in human subjects, where T_3 generated from labelled T_4 appears 12 h after T_4 administration (Silva et al., 1978). T_3 formation is of much greater interest in species in which

the rate of T_4 metabolism is high, namely, in small animals, and especially in birds, where non-hormonal iodinated proteins may disturb T_4 kinetic studies (Astier and Newcomer, 1978).

The present paper describes a procedure which enables one to separate the endogenously generated iodocompounds, without extraction or chromatography, from small sample quantities. When this method is used, no artefact formation should be reckoned with.

Results and discussion

The procedure is based on immunological separation of the iodocompounds. Fifty to 100 μl of plasma originating from consecutive bleedings of experimental animals injected with ^{125}I-labelled thyroxine are transferred into RIA tubes containing 400 μg 8-anilino-naphthalene-sulphonic acid (ANS) in 100 μl barbital buffer, pH 8.4. The samples are then mixed with appropriately diluted, highly specific anti-T_3 rabbit serum, which will bind both ^{125}I-labelled and inactive T_3. After an incubation for 24 h at room temperature, the unbound T_4 molecules present in the system are immobilized with charcoal by centrifugation; the supernatant will contain T_3 + antibody complex and iodine (free, or bound to non-hormonal protein). If the thyroxine disappearance curve is of interest, the procedure can be stopped here with counting the activity of thyroxine in the charcoal fraction. When the T_4 to T_3 conversion rate is to be measur-

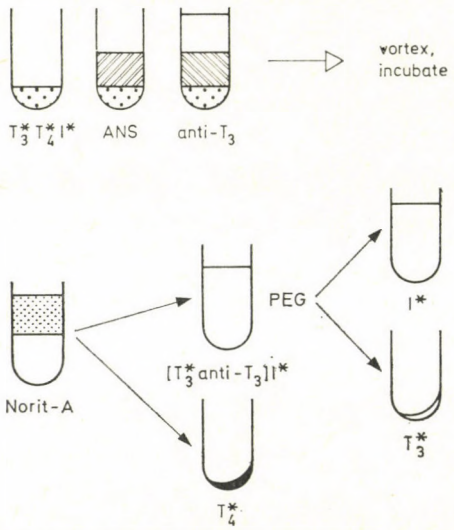

Fig. 1. Demonstration of the process applied to separation of iodothyronines from serum samples by reverse radioimmunoassay (see text)

ed and T_3 quantitation is to be carried out, a further step is necessary. The supernatant formed at the previous step is mixed either with a sufficient amount of goat anti-rabbit IgG or with polyethylene glycol (PEG). As a result of this step anti-T_3 + T_3 complex is precipitated. After centrifugation, the supernatant will contain the iodine alone (Fig. 1). The following points were checked while the method was developed:

(i) Iodocompounds were released from the surface of the transport proteins by means of ANS. The efficiency of that step was checked earlier (Pethes et al., 1978). Four hundred μg ANS per tube was found to be satisfactory in a wide range of hormone concentrations.

(ii) Optimization of the antibody added. To a constant amount (20 pg/tube) of ^{125}I-T_3 increasing quantities (1–5 ng/ml) of unlabelled T_3 were pipetted. The amount of rabbit anti-T_3 was chosen so as to ensure the maximal binding of the highest T_3 quantity.

(iii) The T_4-binding capacity of the charcoal was also studied. In the presence of anti-T_3 added, it was checked whether the purified ^{125}I-T_4 could be precipitated with charcoal when 16 ng/100 μl inactive T_4 was also present in the system. The nonspecific appearance of ^{125}I-T_4 in the supernatant was negligible even when the above-mentioned high concentration of inactive T_4 was present.

(iv) The nonspecific I-binding to charcoal was also investigated with ^{131}I (NaI). Since binding of ^{131}I to charcoal did not occur even in traces, we assumed that all the activity measured in the precipitate originated from iodohormone fraction.

(v) The difference between goat anti-rabbit and PEG separation was also looked at. The disadvantage of the second antibody technique was that the long time needed for incubation (14–48 h) made the procedure unfeasible, and that more than 20% of the ^{125}I-T_3 bound to anti-T_3 remained in the supernatant. The possible reason for this inadequate partition may be the considerable solubility of the complex formed. The separation with PEG according to Cheung and Slaunwhite (1976) allowed a complete immobilization of the anti-T_3 + T_3 complex requiring only seconds for the reaction.

The method, which has been used to investigate the thyroxine metabolism in chickens (*Gallus domesticus;* Rudas and Pethes, 1981), and in Japanese quails (*Coturnix coturnix japonica;* Kovács et al., 1981), is considered applicable especially in T_4 to T_3 conversion studies in connection with investigations into the T_4 metabolic clearance-rate. The advantage of this procedure, in contrast to previous separation techniques, is its specificity and the theoretical exclusion of artefact formation.

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SUBSTRATE AND INHIBITOR SPECIFICITY OF A TRANSIENT, SOLUBLE PROTEOLYTIC ACTIVITY FROM SHEEP RUMEN

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In the rumen liquor supernatant of fistulated sheep a soluble proteolytic factor was found with a peptide-bond preference for the carboxyl-side of lysine and with sensitivity to inhibition by tosyl-lysine chloromethyl ketone (TLCK) and p-chloromercuribenzoate (pCMB). The disappearance of the factor might have been due to changes in the composition of rumen flora.

Keywords. Protease specificity, protease inhibitors, rumen proteolysis, rumen bacteria, sheep.

Ruminants waste dietary proteins because rumen organisms degrade amino acids via "paired" (Stickland-type) deamination to ammonia and volatile fatty acids. Fermentation of proteins has to be preceded by microbial proteolysis.

Several workers have studied the quantitative aspects of ruminal protein degradation, but few investigations have dealt with proteases of selected strains of rumen bacteria (Hunt and Moore, 1958; Lesk and Blackburn, 1971; Hazlewood and Edwards, 1981).

In the present work we approached rumen content as if it were a tissue and we found a proteolytic factor in the supernatant of rumen liquor. Because of its solubility, this factor was much more suitable for investigation than the particle-bound bacterial proteases of the rumen content. Unfortunately, the soluble activity proved to be transient and so it made an end to further studies.

Materials and methods

Rumen content was taken from fistulated Merino sheep fed on grass hay and concentrate. The content was pressed through a layer of cotton to obtain rumen liquor. Bacteria and feed particles were removed by centrifugation at 25,000 *g* for half an hour and the supernatant was used as an enzyme source. Care was taken not to contaminate the supernatant with the bacterial sediment. The feed of the sheep contained no detectable proteolytic activity.

The activity of rumen liquor supernatant was concentrated by acetone precipitation (1 : 1) and centrifugation of the precipitated material. Water residues were extracted by an additional acetone treatment of the precipitate, otherwise activity was rapidly lost in the freezer. The rumen liquor could be stored frozen for at least half a year with little loss of activity, but after acetone precipitation the factor became much more labile in the presence of water.

Proteolytic activity of rumen liquor and its supernatant was determined with azocasein as substrate in 0.1 M pH 6.5 phosphate buffer at 40 °C as described by Baintner (1981). The same method was used to determine the effect of inhibitors. Synthetic substrates were applied under the conditions described in the Tables.

Agarose-bound p-aminophenylmercuric acetate column was prepared according to Sluyterman and Wijdenes (1970).

Results

General proteolytic activity of rumen liquor was approximately 0.6 μg trypsin equivalent per ml; 10–15% of the activity was extracellular, i.e. remained in the supernatant after centrifugation. In individuals devoid of the activity studied, only traces of proteolytic activity were found in the rumen liquor supernatant, while its acetone precipitate was completely inactive.

Little or no inhibition could be achieved with phenylmethyl-sulphonyl fluoride (PMSF) or with tosyl-phenylalanine chloromethyl ketone (TPCK), but p-chloromercuribenzoate (pCMB) and tosyl-lysine chloromethyl ketone (TLCK) inhibited most of the activity. On an organic mercuri column (Sluyterman and Wijdenes, 1970) the factor lost its activity.

Substrate specificity of the factor was determined with a series of synthetic substrates (Tables I and II).

Table I

Substrate specificity of the proteolytic factor in the supernatant of rumen liquor

Substrate	nm	Activity
Benzoyl-L-arginine ethyl ester (BAEE)	253	±
Benzoyl-DL-arginine para-nitroanilide (BApNA)	410	—
Benzoyl-L-tyrosine ethyl ester (BTEE)	256	—
Hippuryl-L-lysine	254	—

Reaction mixture contained 0.1 ml rumen liquor in 2.9 ml 0.1 M pH 6.5 phosphate buffer or 0.1 M pH 8.0 tris-HCl buffer and it was incubated at room temperature in a 3 ml cuvette.

Table II

Peptide-bond specificity of the soluble ruminal proteolytic factor

Substrate	Activity per cent
D-Ile-Phe-*Lys*-pNA	100
Z-Arg-Val-*Leu*-pNA	37
Z-D-Phe-Pro-*Arg*-pNA	16
Z-*Cit*-pNA	0
Suc-Gly-Val-*Gly*-pNA	0
Suc-Gly-Val-*Val*-pNA	0
Suc-Gly-Gly-*Phe*-pNA	0
Suc-*(Ala)*$_3$-pNA	0
Glu-pNA	0

The factor was concentrated by acetone precipitation of the rumen liquor supernatant and incubated at room temperature in a 1.5-ml cuvette. Yellow colour of liberated p-nitroanilide was read at 405 nm. Activities are expressed as % of the highest activity (100%).
pNA = para-nitroanilide; Suc = succinyl; Z = benzyloxycarbonyl.

Discussion

In previous studies (Baintner, 1981), no inhibition of the proteolytic activity of whole rumen liquor could be achieved by addition of ethylene diamine tetraacetic acid (EDTA) or by inhibitors of soybean, plasma, egg white or lamb's meconium. In the present work we examined the effect of additional inhibitors on the proteolytic activity of the rumen liquor supernatant. The sensitivity to inhibition by pCMB indicates that the factor contained proteases requiring sulfhydryl group(s) for function. On the other hand, the marked, but not complete, inhibition by TLCK suggests that a trypsin-like serine protease was the main component of the factor. However, PMSF, a specific inhibitor of serine proteases, was ineffective, maybe due to its decomposition during the rather long incubation period. We failed to identify the factor with known proteases.

The Tables show that the soluble proteolytic factor of rumen liquor preferred the substrate containing L-lysine to the substrates containing L-arginine or other amino acids. (Hippuryl-L-lysine is an exception, for this substrate, being specific for carboxypeptidase B, is not split by proteases). Most of the substrates were not split at all. The preference for lysine is in agreement with the marked sensitivity of the factor toward TLCK, an inhibitor containing lysine.

After the unexpected disappearance of the activity under study from the rumen of our experimental sheep, only traces of activity remained in the super-

natant, almost all of the activity being in the particulate fraction of the rumen liquor. The residual activity of the supernatant could not be inhibited by TLCK. We failed to restore the original condition by various dietary changes and to find the factor in other fistulated sheep. The disappearance of the factor might be caused by an accidental elimination of a transient bacterial species from the rumen.

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OCCURRENCE OF TRYPSIN INHIBITORS IN COLOSTRUM, MECONIUM, AND FAECES OF DIFFERENT SPECIES OF UNGULATES AND CARNIVORES

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Trypsin inhibitor concentrations were determined in colostrum, milk, meconium and faecal samples taken from different species of ungulates and carnivores.

High but declining inhibitor contents were found in the *faeces* of young calves and lower concentrations in the faeces of suckling lambs.

Until data about more species become available, only preliminary conclusions can be drawn in respect of the relationship between taxonomy and the occurrence of trypsin inhibitors: a) Artiodactyla (ruminants and sow) and Carnivora secrete acid-resistant trypsin inhibitor with the *colostrum*. b) *Meconium* trypsin inhibitor appears to be a characteristic of ungulates, although the diminishing fetal values approximate zero in the newborn piglet. c) Data for *faecal* trypsin inhibitor are available in young ruminants only.

Keywords. Trypsin inhibitor, colostrum, meconium, faeces, newborn, taxonomy, ungulate, carnivore.

Laskowski and Laskowski (1951) discovered and crystallized from bovine colostrum a trypsin inhibitor that was resistant to 2.5% trichloroacetic acid and inhibited α-chymotrypsin, too. A similar inhibitor was found in the colostrum of the sow (Laskowski et al., 1957), ewe (Baintner, 1976; Sandholm and Honkanen-Buzalski, 1979) and cat (Baintner, 1973). The low-level acid-sensitive trypsin inhibitor of human colostrum (Barkholt-Pedersen et al., 1971) and rat milk (Weström and Carlsson, 1976) is derived from the serum and is mainly α_1-antitrypsin.

In another line of investigations a trypsin inhibitor factor was discovered in the meconium of the pig fetus; this inhibitor disappeared around term (Carlsson and Karlsson, 1972; Baintner, 1975). High inhibitor content was found in the meconium of the newborn lamb (Baintner, 1975), and the inhibitor was partially purified from it (Baintner, 1980). The factor inhibited trypsin, α-chymotrypsin, elastase and subtilisin. It also occurred in the faeces of the suckling lamb in low and gradually diminishing concentrations (Baintner, 1980). Human meconium lacks trypsin inhibitor; cat's meconium contains low levels of proteases (Baintner, 1975).

In contrast to trypsin inhibitors of the plasma, the inhibitors of colostrum and meconium do not occur in all mammalian species; therefore, it was of interest to examine as many species as we could do.

Materials and methods

Colostrum samples were taken by milking them into test tubes or capillary tubes, using oxytocin stimulation for the small animals. Usually one meconium or colostrum sample was taken from each animal. The time elapsed between parturition and sampling was noted and used for age designation. The reindeer, blue fox and ferret colostrum samples were dried on glass plates at room temperature, then scraped into test tubes and redissolved before use. Colostrum samples of other species were stored in freezer. Faecal and meconium samples were dissolved in a large excess of saline, but complete dissolution could not be achieved with meconium.

Trypsin and chymotrypsin were determined with synthetic substrates by measuring the increase of extinction in the UV range of a spectrophotometer. Benzoyl-arginine ethyl ester (BAEE) and benzoyl-tyrosine ethyl ester (BTEE) was used as substrate for trypsin (Schwert and Takenaka, 1955) and chymotrypsin (Hummel, 1959), respectively. Trypsin inhibitor values were calculated from the decrease in the activity of a known amount of trypsin in the presence of an aliquot of sample. Results were expressed as inhibited trypsin milligrams or micrograms and were related to ml or gram of dry matter of the faecal sample, and of the wet meconium. The dry matter content of meconium had been found to be rather constant (about 85%). The samples were also examined for chymotrypsin inhibitor content, but no numerical data are given because the error was much higher than with the determination of trypsin inhibitor.

Mean and standard deviation were calculated when the number of samples was sufficient.

Results

A) *Trypsin inhibitor in colostrum and milk*

Mare: The five samples of colostrum (from 0 to 1/2 day) contained traces of inhibitor. Resistance to acid was not determined because of the low values.

Dwarf goat: The four colostrum samples markedly inhibited the activity of trypsin. No quantitative studies were made.

Reindeer:

2 h	1.6	mg/g dry matter
10 h	0.9	
1/2—1 day	0	
1/2—1 day	0	
Mature milk	0	

Cat: Each of the eight milk samples (from 1/2 to 6 days) contained trypsin inhibitor: $\bar{x} = 105 \pm 30$ μg/ml.

Bitch (German sheep-dog): 0 h 2.5 mg/ml
6 h 2.4 mg/ml
12 h 0.2 mg/ml
Blue fox: One sample (between 1 and 2 days), 4.3 mg/g
Ferret: One sample (about half day), 3.1 mg/g

B) *Trypsin inhibitor in meconium*

Values are related to wet weight.
Foal: 1.6 mg/g; 1.4 mg/g; 1.3 mg/g.
Dwarf goat kid: 6.6 mg/g; 4.4 mg/g; 3.4 mg/g.
Calf: Six samples; mean 16.0 $\pm$ 7.0 mg/g; range between 26.7 and 7.9 mg/g.
Reindeer calf: Seven samples; mean 10.0 $\pm$ 1.0 mg/g; range between 33.5 and 1.6 mg/g.

C) *Trypsin inhibitor in faeces*

Reindeer calf: The sample taken on the 4th day served as a control; it lacked inhibitor, but contained significant trypsin and chymotrypsin activities.
Calf: Results are related to dry matter.
a) Holstein-Friesian calves: 5.5 $\pm$ 0.8 mg/g; n = 5; 3 days.
b) Holstein-Friesian calves: 6.3 $\pm$ 2.6 mg/g; n = 8; 10 days.
c) Holstein-Friesian x Hungarian Spotted F_1 calves:

Age, days	Concentration, mg/g	n
1— 2	8.5	4
2—10	7.2 $\pm$ 3.1	6
10—35	5.1 $\pm$ 2.4	8
35—42	0.21 $\pm$ 0.14	8
42—50	0.20 $\pm$ 0.05	5
50—60	0.17 $\pm$ 0.11	8
~100	0	2

Discussion

Although our data are scarce for most of the species, we decided to publish even single values for two reasons: 1) it is difficult to collect colostrum and meconium samples, because the owners of the animals are usually anxious about the possible interference with the essential mother–offspring relation; 2) even single values fitted well into trends within taxonomic groups when we compared the present findings with literary data (Table I).

Table I

Occurrence of acid-resistant trypsin inhibitors in different mammals

			Colostrum	Meconium	Faeces
		Man	— (Barkholt-Pedersen, 1971)	— (Baintner, 1975)	—
		Rat*	—	—	—
		Rabbit*	—		
Ungulata	Perissodactyla	Horse	Nothing or in traces	+	
	Artiodactyla	Pig	+ (Laskowski et al., 1957)	Decreasing values	—
	Ruminantia	Cattle	+ (Laskowski et al., 1951)	+	+
		Sheep	+ (Baintner, 1976)	+	+
		Goat (dwarf)	+	+	
		Reindeer	+	+	— ?
	Carnivora	Cat	+ (Baintner, 1973)	—	
		Dog	+		
		Ferret	+		
		Blue fox	+		

* Unpublished observations.

All samples that contained trypsin inhibitor in significant concentration inhibited α-chymotrypsin, too.

Acid-resistant colostral trypsin inhibitor appears to be a characteristic of carnivores and Artiodactyla, i.e. ruminants and pig (Table I). Low inhibitor concentration was found in mare's colostrum (Perissodactyla) by Sandholm and Honkanen-Buzalski (1979) and traces in the present investigation. We had no first colostrum from the cat, while all the cat's milk samples contained low but significant concentrations of acid-resistant inhibitor.

Trypsin inhibitor content of the colostrum of carnivores seems to be higher than that of ruminants, while human (Barkholt-Pedersen et al., 1971), rat (Weström and Carlsson, 1976) and rabbit colostrum (Baintner, unpublished) lack acid-resistant inhibitor.

Meconium trypsin inhibitor appears to be a characteristic of ungulates (Table I), although diminishing fetal values approximate zero in the newborn piglet (Carlsson and Karlsson, 1972; Baintner, 1975); it is lacking from the meconium of cat, rat and man (Baintner, 1975).

Faecal trypsin inhibitor has been found in the young lamb (Baintner, 1980) and even more in the young calf (present studies), but not yet in other species. The inhibitor completely disappeared from the faeces at or before physiological weaning time. In the lamb, meconium and faecal inhibitor factors showed similar characteristics (Baintner, 1980).

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RAPID DETERMINATION OF MONENSIN AND NIGERICIN IN MEDICAL PREMIXES AND IN GROWTH-PROMOTING FEED PREPARATIONS

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A quantitative thin-layer chromatographic method has been developed for determination of the active ingredient content (0.05 to 10%) of medical premixes and growth-promoting feed preparations containing nigericin and monensin. The antibiotics were dissolved in methanol at room temperature. Chromatography was performed on a silica gel layer with a 9 : 1 chloroform–methanol mixture. The intensity of spots, developed by vanillin–sulphuric acid solution, was measured with a spectrodensitometer. The results are in good agreement with data obtained by the microbiological method. The error of the measurement was ±5% with 90% probability on the basis of 5 to 8 parallel measurements.

Keywords. Monensin, nigericin, quantitative thin-layer chromatography, determination, premix.

Among the polyether-type antibiotics, monensin (Agtarap and Chamberlin, 1967; Agtarap et al., 1967; Chamberlin and Agtarap, 1970; Haney et al., 1967) and nigericin (Harned et al., 1951; Horváth and Gyimesi, 1971; Steinrauf et al., 1968; Stempel et al., 1969) are widely used in veterinary practice as active components of medical premixes and growth-promoting feed preparations.

In fowls, monensin-sodium is used against *Eimeria* species because of its excellent coccidiostatic effect (Chapman, 1974; Fitzgerald, 1972). It has been shown in beef cattle that, as a result of its specific effect on the intestinal flora, it promotes transformation of feedstuffs to energy (Brown et al., 1974; Oliver, 1975; Raun et al., 1974; Richardson et al., 1974), and therefore it is a component of growth-promoting feed preparations.

Nigericin is used therapeutically in pigs with enzootic pneumonia and dysentery and in turkeys suffering from sinusitis. This drug is efficient in such cases until *E. coli* becomes the dominating flora. The main advantage of the veterinary use of both drugs is that they are not used in human therapy.

Microbiological (Breunig et al., 1972; Breunig et al., 1977; Kavanagh and Willis, 1972; Kline and Rathmacher, 1969; Smither, 1975), spectrophotometric (Golab et al., 1973), paper and thin-layer chromatographic methods with bioautographic (Amman and Gottlieb, 1955; Begue and Kline, 1972; Donoho and Kline, 1967; Haney et al., 1967) and in situ detection are used for the determi-

nation of monensin in fodders and premixes (Koufidis, 1976). Nigericin residues have been measured by microbiological methods in animal tissues (Czeglédi-Jankó and Dankó, 1980).

In premixes trace amounts of antibiotics can be measured rapidly by thin-layer chromatography with the same accuracy as by microbiological methods. The chromatographic method is suitable for the detection of degradation products, too, in addition to the active component; thus, it is an efficient tool for investigation of stability.

Materials and methods

Monensin-sodium was obtained from Eli Lilly and Co. Nigericin-sodium was a three times recrystallized BIOGAL product. Its active ingredient was checked by potentiometric titration. The chemicals and solvents used in the experiments were analytical-grade products.

The antibiotics were dissolved from premixes and feed preparations with methanol at room temperature.

Chromatography was performed on a Merck 5721 silica gel layer without preliminary treatment of the layer, using a 9 : 1 chloroform–methanol mixture.

Spots of nigericin and monensin were developed by a vanillin–sulphuric acid solution (3 g vanillin was dissolved in 100 ml ethanol and 1.5 ml concentrated sulphuric acid was added). The layers treated with the developing solution were heated at 80 °C for 5 min.

Quantitative analysis of the data was performed with a Shimadzu CS-920 spectrodensitometer at $\lambda = 505$ nm (for monensin) and $\lambda = 510$ nm (for nigericin) wavelengths.

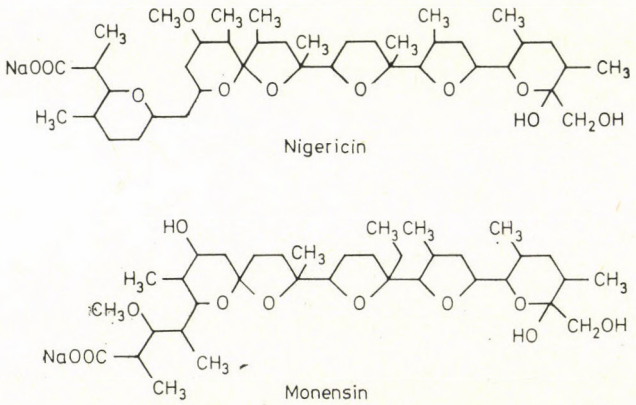

Fig. 1. The structure of nigericin and monensin

Results and discussion

The usual formulation of chemicals which are effective in low concentration for increasing growth and for therapeutical purposes is that the active ingredient is mixed in a feedstuff containing minerals, vitamins and proteins.

The active ingredient should be dissolved from the matrices, or at least it should be concentrated for the analysis when any of the analytical methods is used. Although nigericin and monensin contain carboxylic groups, they are well-soluble, primarily in organic solvents, among which methanol was found to be the most suitable; during a 5-min digestion the antibiotics are completely dissolved as proved by control measurements. Some other components of the base-premix also appeared in the methanol extract, but they could be either separated from the antibiotics by chromatography or, because of the selective development, they do not disturb the measurement of the main component. It is advisable to filter the methanolic solution before application for chromatography.

Five to 10 μl of the filtered solution (which is equivalent to 10 to 20 μg nigericin or monensin) was applied to the layer in small portions dried with hot air. Chromatography was carried out using a 9 : 1 chloroform–methanol mixture in a chamber saturated with vapours of the eluent. The R_f values obtained for the antibiotics are as follow:

R_f, monensin	0.9
R_f, nigericin	0.7
R_f, deoxy-nigericin	0.55

The relation between the quantity of antibiotics applied on the layer and the detector signal of densitometer is linear in the range from 0 to 30 μg, using the first-order linear programme (LIN 1) of the instrument.

In routine measurements, two standard quantities were applied to all layers which surrounded the quantities of antibiotics in the samples. The intensity of the red spots of nigericin and monensin was unchanged for two hours after development.

Mixtures containing nigericin

The accuracy of the thin-layer chromatographic method and the possible disturbing effect of matrices have been studied by the analysis of mixtures with known composition. Analysis of two different products was performed: premix A contained only potato starch in addition to 2% of nigericin. The nigericin content of premix B was 2% as well, but the matrix was more complex, containing minerals, vegetable flours and vitamins. The results are summarized in Table I.

Table I

Analysis of nigericin-containing premixes

	Nigericin content, %	Control measurement, %	Standard deviation, %	P	n
A	2.0	98.2–101.9	±0.8	0.90	7
B	2.0	95.1–104.5	±4.0	0.90	6

The results clearly show that the accuracy and the reproducibility of the quantitative thin-layer chromatography are excellent. The more significant but not systematic errors occur in the system containing more complicated matrices.

Mixtures containing monensin

The matrices of all mixtures were similar to premix B. A part of the results was compared to the data obtained by the agar-diffusion microbiological method and a good agreement was observed in this case. In other cases the monensin content was known from loading, and the results are given as percentages of loading, too (Table II).

Table II

Investigation of monensin-containing mixtures

	Monensin content, %	Microbiological determination, %	TLC result, %	Standard deviation, %	P	n
Rumensin®	10.0	10.87	10.89	±3.5	0.90	7
		11.09	11.35	±1.8	0.90	7
Beef cattle premix	0.2	0.197	0.195	±5.1	0.90	5
		0.198	0.201	±3.5	0.90	5
Fattened lamb premix	0.055	0.053	0.055	±3.6	0.90	8

TLC = thin-layer chromatography

The results clearly prove that a 0.05 to 10% monensin content can be assayed in premixes by quantitative thin-layer chromatography with the same accuracy as by microbiological methods. Quickness and simplicity are the advantages of the chromatographic method.

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BOOK REVIEW

DENNY, H. R.: *Orthopädische Chirurgie am Hund.* Ferdinand Enke Verlag, Stuttgart, 1983. 203 pages, 365 figures. Retail price: 26.80 DM. Original title: A Guide to Canine Orthopaedic Surgery. Blackwell Scientific Publ., Oxford, 1980. Translated and the German edition revised by K. Bonath and M. L. Nagel.

The last decades have seen a remarkable progress in all fields of veterinary surgery. The progress compels both teaching staff and veterinary practitioners to obtain new information and to take part in a continuous, active extension training. This book offers a possibility for the Reader to attain up-to-date information representing the present state of knowledge on canine orthopaedic surgery.

The author discusses the essence of orthopaedic diseases. Comprehension is greatly facilitated by the large number of figures. Primarily information serving for better performance of the daily routine work is provided. This, of course, implies the disadvantage that the diseases are not discussed in full detail. However, the abundant list of references given at the end of chapters counterbalances this drawback and provides ample information for the Reader.

The book is divided into five chapters. The first chapter deals with *bone fractures* and, in addition to the characteristics of fractures, discusses the healing and radiographic diagnosis of fractures, fracture-induced disorders in bone growth, bone transplantation techniques as well as joint, tendon and muscle injuries.

The theme of the second chapter is the *treatment of fractures.* After discussing the conservative management of fractures, the author gives a detailed account of the principles and methods of surgical treatment, and of the different metal implants, including the optimal conditions of their removal.

The chapter dealing with *diseases of the skull and vertebral column* contains information on fractures of cranial and jaw-bones and their treatment, luxation and subluxation of the mandibular joint, and mandibular neuropraxia (so-called paralysis of the trigeminal nerve). The diagnosis of vertebral column diseases is facilitated by the description of valuable and novel neurological examinations. The chapter provides also a stimulating account of the dislocation and fracture of spinal vertebrae and of the treatment of fractures by osteosynthesis.

The last two chapters deal with orthopaedic diseases of the *fore-* and *hind-limbs*, respectively. Not only a brief but very practicable description of bone, joint, tendon and muscle diseases is provided, but also a survey of developmental abnormalities and consequences of growth and metabolic disturbances. Injuries of the peripheral nerves supplying the limbs are discussed in association with diseases of the vertebral column.

Most of the information contained by this book has been available so far only in scattered form, as part of manuals, atlases, research articles, papers read at conferences and extension courses. The author deserves praise for having critically evaluated and systematized the pertinent professional knowledge, left out controversial or insignificant information, thus having created a book extremely useful for the practice. For the easily comprehensible text both the author and the translators deserve praise.

I. SZOKOLÓCZY

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Acta Veterinaria Hungarica 32 (3—4), pp. 105—116 (1984)

NUTRITIVE VALUE OF SLAUGHTER BY-PRODUCT MEALS

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(Received June 13, 1984)

Slaughter by-product meals are produced from confiscated organs, blood, feather, poultry legs and heads, guts, etc., in dry rendering plants by cooking, drying, and partial defatting. The wide variation in composition and nutritional value of such products is attributed to the variation in the nature of the raw materials and the conditions used in their processing.

The raw materials vary in protein, fat and ash content and the quality of the protein is determined by the type of soft and hard offals used.

The nutritional value of meat-and-bone meals is unchanged over a wide range of processing times and temperatures. However, after prolonged heating at extreme temperatures, the availability of essential amino acids may decrease markedly. Toxins from oxidized fats or bacterial contamination have in the practice little effect on the nutritional value of meat-and-bone meals.

The slaughter by-product meals are rich in lysine and thus can complement cereals.

Keywords. Meat-and-bone meal, blood meal, feather meal, nutritive value, protein quality.

Bones and soft animal tissues have long been used in the manufacture of meat-and-bone meals. These products are nutritionally and economically important sources of protein for livestock, particularly for poultry and swine.

They are primarily a rich source of protein, but the contribution of minerals, vitamin B complex and energy are also important. The slaughter by-product meals are usually dried products from abattoirs and, depending on the raw material, are called meat meal, bone meal, meat-and-bone meal, blood meal, or feather meal. From these products meat-and-bone meals are produced in the largest quantity from animal carcasses and inedible slaughter by-products, such as confiscated organs, offals, blood, poultry legs and heads, guts, etc.

The importance of recycling of the losses of slaughtering to the animal production is well indicated by the quantity of raw materials available for rendering in Hungary, which is more than 200 thousand tons per year.

The large variation in composition and growth-promoting ability of meat-and-bone meals from the same and from different rendering plants have been recognized and widely reported for many years. In the present paper a review based on our experiences is reported on the factors which can influence the nutritive value of animal protein meals.

Materials and methods

Production and origin of animal by-product meals

The fundamental process in the production of meat-and-bone meals is heating of raw materials, removal of the moisture, and partial defatting (Berzsenyi, 1978; Regős and Berszán, 1978). The raw by-products obtained from slaughterhouses are heated in dry rendering plants to the point of sterilization. In the dry rendering method, the steam used for heating does not come into contact with the raw material (Szél and Gál, 1980; Hussel and Kiehn, 1980).

The typical losses during the slaughtering of swine and beef cattle, the raw material basis of meat-and-bone meal production, are shown in Figs 1 and 2 (Solymos and Krajcsovics, 1979).

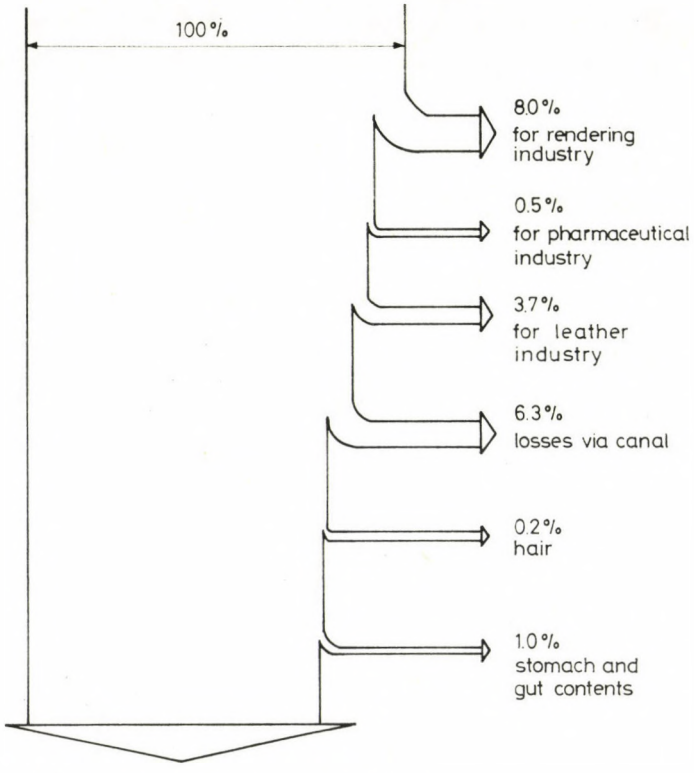

Fig. 1. Losses during swine-slaughtering

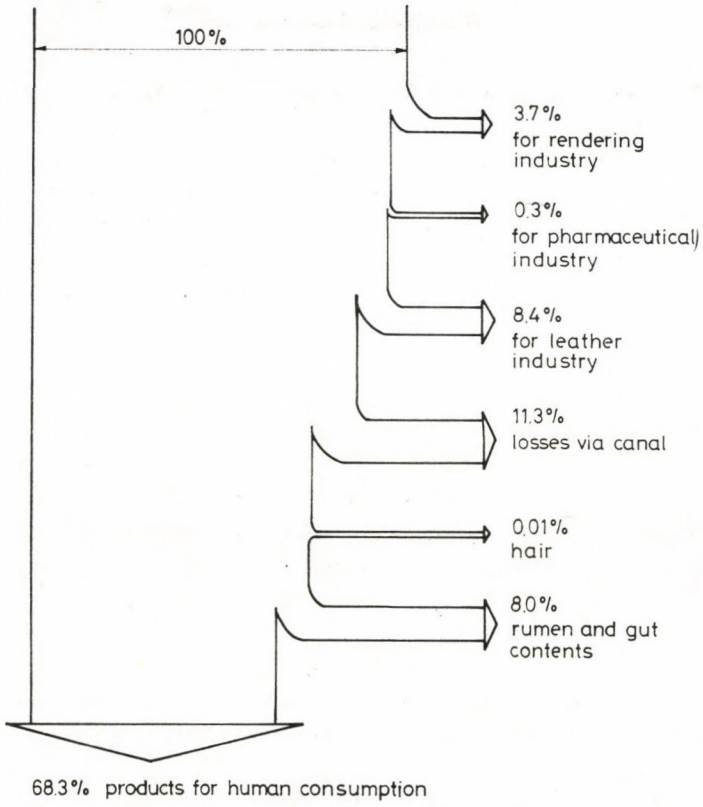

Fig. 2. Losses during beef cattle slaughtering

The animal protein meals examined (meat-and-bone meal, poultry by-product meal, blood meal, feather meal) were produced in different dry rendering plants on an industrial scale.

Methods

Chemical composition of the samples was determined according to the Hungarian standard MSZ 6830[1].

The net protein utilization (NPU) was measured with the carcass method, which determines the difference in carcass nitrogen between rats fed a test protein, and those fed a protein-free diet (Hegedűs et al., 1982, 1983*a*).

Amino acid analyses were performed in an ion-exchange liquid chromatograph (Hegedűs et al., 1983*b*). Hydroxyproline was determined by the colour reaction of oxidized hydroxyproline with p-dimethylaminobenzaldehyde (Hegedűs and Bokori, 1983).

[1] MSZ 6830 (1981): Takarmányok táplálóértékének megállapítása. Szabvány, Budapest.

Results and discussion

Chemical composition of meat-and-bone meals

The wide variation in chemical composition and nutritional value of meat-and-bone meals is attributed to the variation in the nature and composition of the raw materials and the conditions used in the processing (Eggum, 1970). There is evidence that the raw materials used in the manufacturing of meat-and-bone meals can largely affect the nutritive value of these products. Similarly, the processing conditions used in dry rendering plants may also be important in determining the growth-promoting ability of meat-and-bone meals.

The moisture and fat content of meat-and-bone meals can be standardized by processing (drying, fat extraction). Thus the crude protein content and ash content will depend on the composition of the raw materials used.

The typical chemical composition of meat-and-bone meals according to the Hungarian standard is shown in Table I.

Table I

Chemical composition of meat-and-bone meals according to the Hungarian standard

	Types of meat-and-bone meals	Moisture (%) (maximum)	Crude protein (%) (minimum)	Crude fat (%) (maximum)	Crude ash (%) (maximum)
I	(a)	10	62	15	22
II	(a)	10	58	15	28
III	(b)	10	54	15	28
IV	(b)	10	50	15	30
V	(b)	10	46	15	34
VI	(solvent extracted) (b)	10	54	8	30
VII	(solvent extracted) (b)	10	50	8	34

(a) = No keratinous raw material is permitted
(b) = Crude protein can contain maximum 10 per cent keratin

The crude protein content is in general in significant negative correlation with the crude ash content. This is illustrated in Fig. 3 (Hegedűs et al., 1984*a*).

The effect of raw materials

In meat-and-bone meal the crude protein level is in inverse relation, while the ash content is in direct relation with the proportion of bone in the raw material (Figs 4 and 5).

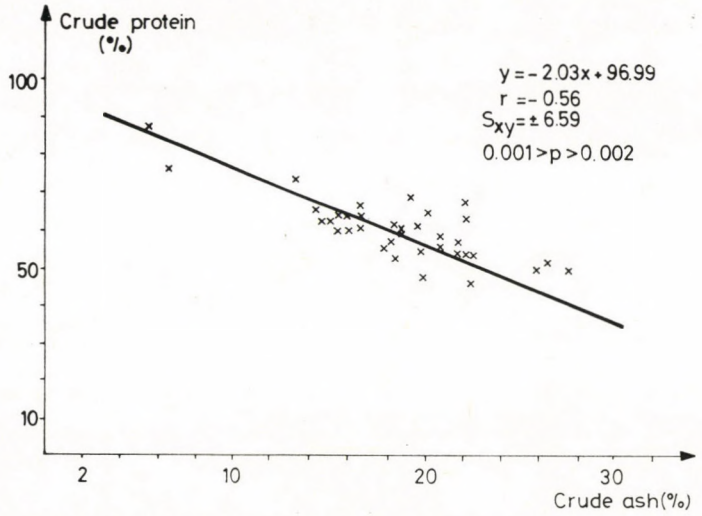

Fig. 3. Correlation between crude protein and ash content of meat-and-bone meals

Bones contain large amounts of collagen which has a low nutritional value due to deficiencies of several essential amino acids, particularly of tryptophan (Hegedűs et al., 1983*a*).

The proteins of the connective tissues are relatively rich in hydroxyproline, as compared to other meat proteins. The net protein utilization (NPU-index) of meat-and-bone meals decreases with the increasing amount of hydroxyproline in the meals. When the NPU value was plotted against the amounts of

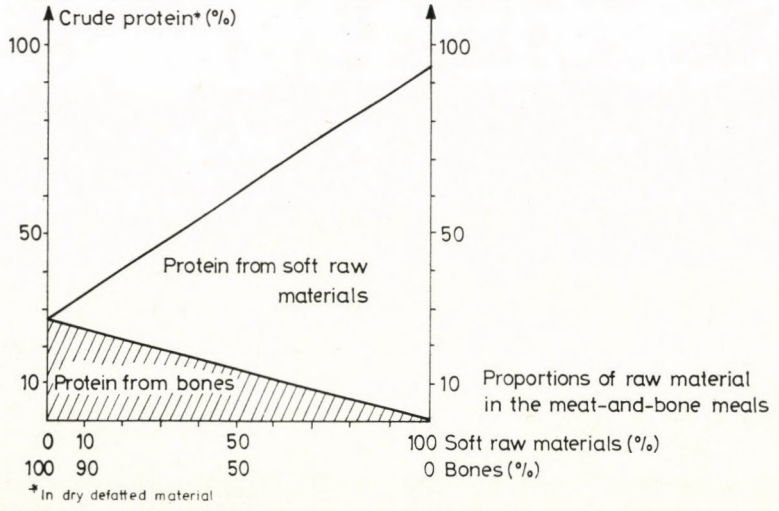

Fig. 4. Contribution of bone and soft raw material to the protein content of meat-and-bone meals

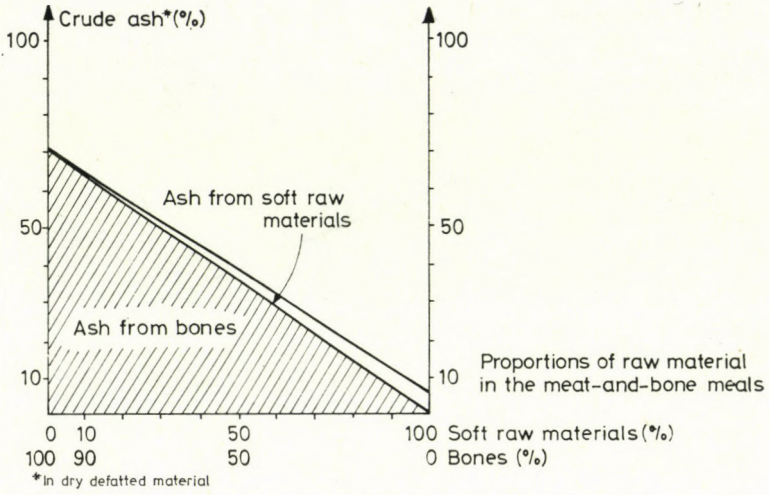

Fig. 5. Contribution of bone and soft raw material to the ash content of meat-and-bone meals

hydroxyproline in log (g/16 g N), the negative correlation was more pronounced. Thus, hydroxyproline content has a certain value for predicting the net utilization of proteins in meat-and-bone meals containing raw materials rich in collagen (Fig. 6; Hegedűs and Bokori, 1983).

Meat-and-bone meals are virtually carbohydrate-free and only their protein, lipid, mineral and vitamin contents have to be considered. From these, the proteins are the most important nutrients (Hegedűs et al., 1984*b*).

Atkinson and Carpenter (1970) studied the influence of raw materials on protein quality of meat-and-bone meals produced from bovine muscle,

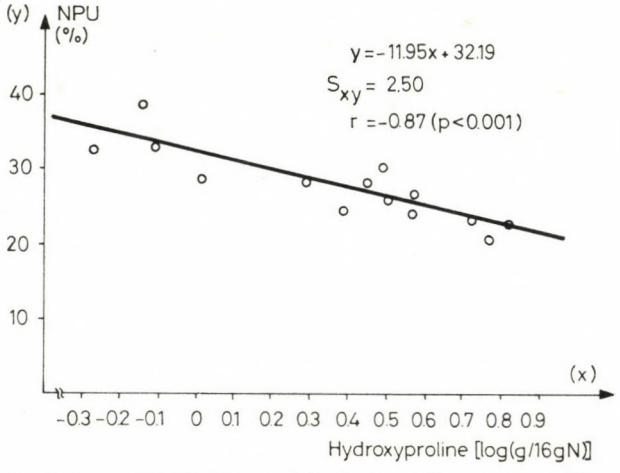

Fig. 6. Regression between NPU value and hydroxyproline content of meat-and-bone meals

gut and tendon. The dilution of muscle protein of high nutritive value with tendon and ossein of low nutritive value has resulted in lower NPU values.

Meat-and-bone meals, containing a certain amount of skin, cartilage and connective tissue, have a high collagen content. Collagen has been shown by Bender and Doell (1957) to have no biological value for rats fed as sole source of protein in a test diet.

The effects of different raw materials and processing conditions used in the manufacture of meat-and-bone meals was studied by Skurray and Herbert (1974); significant correlation was found between weight gains or feed conversion efficiencies of chicks and the collagen content of the various materials even with different processing times and temperatures.

Blood proteins, in which meat-and-bone meals are usually poor, are deficient in isoleucine; when used as the main source of protein in rat diets, they may depress growth (Hegedűs, 1983).

The predominant protein of hoof, hair, wool and feather is keratin. The nutritive value of keratin as the sole source of protein in experimental chicken or rat diets is very low. This has been attributed to the usually low digestibility of keratin and to a deficiency in keratin of several essential amino acids (viz. histidine, methionine, lysine and tryptophan). The high cystine content of adequately processed feather meal, however, can be utilized efficiently (Hegedűs, 1984).

Table II

Effect of different raw materials on the feed intake, growth, and net protein utilization of rats (Hegedűs, 1981)

Raw material (sole protein source in test diet)	Feed intake (g/rat/10 days)	Average weight gain in 10 days (g)	NPU (%)
Pig stomach	29.9	0.3	16
Beef tendon	34.4	0.2	5
Beef vein	38.0	3.9	18
Pig meat cuts	79.0	32.5	64
Residues of fat rendering	70.7	17.5	49
Meat residues, separated from raw bones	68.0	21.0	51
Pig skin	25.4	−5.7	0

Average initial weights of rats were 55.2 ± 0.1 g.
Crude protein levels of test diets were 10 per cent.
Protein sources were lyophilized and ground.

The effect of different raw materials on the feed intake, growth, and net protein utilization of rats is illustrated in Table II (Hegedűs, 1981).

The effect of processing conditions

The most important processing in the manufacture of meat-and-bone meals is the *heat treatment.* Its effect can be both beneficial and detrimental. Heat destroys undesirable microorganisms, enhances digestibility and increases palatability. The undesirable effects of thermal processing are destruction of amino acids by oxidation, modification of some of the linkages between the amino acids that are either not hydrolysed during digestion or that the release of amino acids is delayed (Lang, 1970; Nesheim, 1974).

There is much evidence in the literature that the usual *cooking* time and temperature (30 min at 130 °C) generally have little effect on the nutritive value of meat-and-bone meals (Bender, 1972). During *prolonged heating*, however, the nutritive value of meat protein may fall. Donoso et al. (1962) heated pork *in water* at 110 °C for 24 h, then dried it at 100 °C for 16 h; NPU fell from 76 to 41, available lysine fell by 30%, and 16% of the methionine and 44% of the cystine were destroyed.

Blood meals are especially sensitive to heat treatment. Satisfactory blood meal (available lysine content 5% or greater) can be produced for use in feedstuffs, if processing temperatures remained under 135 °C (Hamm and Searcy, 1976). New processes (spray drying, flash drying, ring drying, continuous process) are very promising in this respect (Kramer et al., 1978).

Overheating in the processing of raw materials of meat-and-bone meals causes profound changes in the protein molecules. The structural changes lead to a retardation of enzymatic digestion of proteins. The effect is a consequent reduction in the amount of essential amino acids released for absorption into the bloodstream. Lysine is involved in the formation of peptide linkages by its side-chain amino groups. Under severe heat treatment this reaction can proceed to a point where the availability of lysine becomes very low (Hegedűs et al., 1981).

Drying can also affect the availability of amino acids. As processing proceeds, water evaporates and the decrease in the latent heat of evaporation allows the temperature of the meat by-products to increase rapidly (Skurray, 1982), resulting in a marked decrease in the availability of amino acids. Overdrying to a moisture content of less than 5 per cent enhances the hydrophobe character of by-product meals, and their digestibility becomes lower.

Protein quality of meat-and-bone meals

The variation in the nutritional value of the protein is not only due to the changing protein content of different by-product meals but also the different concentration of indispensable amino acids and their availability.

The quality of proteins of meat-and-bone meals depends on several factors, such as

— digestibility,
— amino acid composition,
— available amino acid content,
— possible toxic factors.

The incomplete availability of essential amino acids in meat-and-bone meals is attributed to the undigestible nature of some of the raw materials used in their manufacture. The digestibility and nutritive value of many animal proteins have been shown to improve on denaturation by heat. Prolonged heating at high temperature, however, may lead to heat damage of meat-and-bone meals (Hegedűs et al., 1981).

In the quality control of meat-and-bone meals the crude protein content determined by the classic Kjeldahl procedure can be misleading in the prediction of the nutritive value. This is particularly the case when raw materials containing keratin, e.g. feathers, hair, hooves, are also used. The digestibility of feather-protein is low if it is not adequately denaturated. At the same time, the protein content of feather is high. In such cases, the crude protein content of meat-and-bone meals is much higher than their available protein content (Fig. 7, Bokori and Hegedűs, 1980). To eliminate this problem, raw materials with high keratin content must separately be processed and put into circulation.

To control the quality of proteins of meat-and-bone meals, not only the pepsin-soluble-nitrogen and the amino acid composition must be tested, but rather the availability of amino acids.

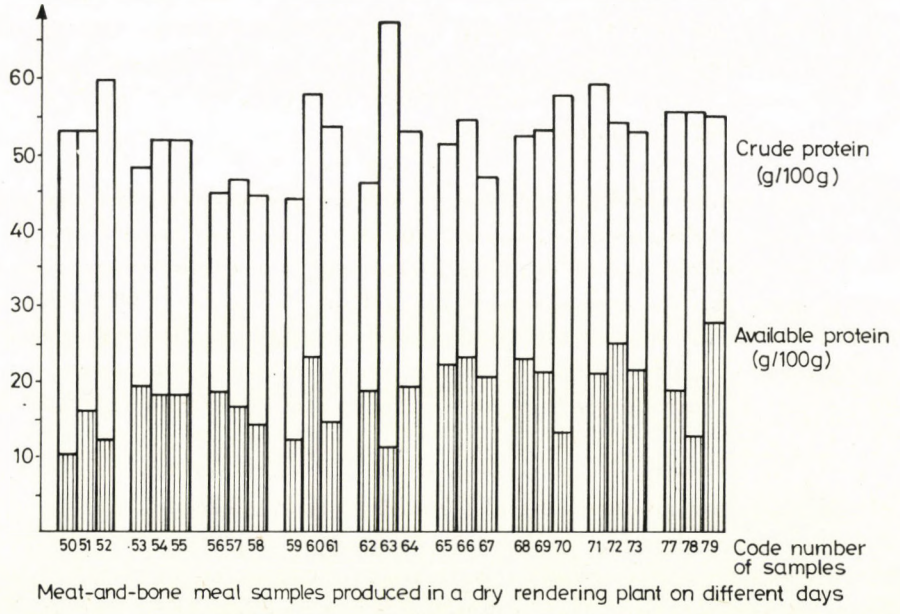

Fig. 7. Crude- and available protein content of meat-and-bone meals produced from raw materials containing a varying proportion of feather

Meat-and-bone meals having surplus in lysine are used to complement cereal grains, in which lysine is the limiting essential amino acid. For this reason, the available lysine content is an important parameter to indicate the growth-promoting ability of meat-and-bone meals.

Available lysine content of different by-product meals determined by the rat-growth test varied considerably. Especially the blood meal samples examined by us had low available lysine contents (2–3 g/16 g N). The available lysine content of all the examined protein meals (meat-and-bone meals, fish meals, blood meals, poultry by-product meals, feather meals) was considerably lower than their gross lysine content (Hegedűs et al., 1982).

The growth rates of rats on meat-and-bone meal based diets are sensitive to both the amount of meat-and-bone meal and the types of cereals in the diet. The protein quality of mixtures of wheat and meat-and-bone meal, barley and meat-and-bone meal, as well as maize and meat-and-bone meal, characterized by in vivo indices (rat-NPU, relative weight gain, feed efficiency) showed that in all of the feed ingredient pairs studied, the mixtures exhibited complementary effects. However, in vitro indices (limiting essential amino acids, sum of essential amino acids, Chemical Score, Essential Amino Acid Index, Predicted Value) have failed to detect in several cases the complementary effects shown by in vivo indices (Hegedűs et al., 1983*b* and 1983*c*).

Possible toxic factors

The possible growth-depressing factors present in meat-and-bone meals are considered to be

— toxins from oxidized fat or Maillard reaction,
— bacterial contamination,
— high calcium levels.

Toxins from rancid fats or toxic products of Maillard reaction as well as toxic decomposition products (biogen amines) are of minor importance.

There has been considerable discussion on the possible danger associated with the presence of oxidized fats in animal diets (Skurray, 1982). However, it has been shown that, using an oxidized meat-and-bone meal sample, it was practically impossible to formulate a diet which had a peroxide value high enough to cause growth depression in poultry (Atkinson and Carpenter, 1970).

The quality of fat in meat-and-bone meals should not be overemphasized because of the low concentration of peroxide in the final feeds.

Concerning the Salmonella contamination of meat-and-bone meals, it is generally expected that with modern factory practices of cleanliness and a high enough rendering temperature bacterial toxicity will not arise (Nyiredy, 1963; Skovgaard, 1980).

When very high levels of meat-and-bone meal are used as a protein supplement to cereal diet the excessive dietary calcium levels (up to 2.5% Ca in the feed) may cause depression in chick growth. In the practice, however, such a high proportion of meat-and-bone meal is not used (maximum 4–10% meat-and-bone meal in the feed). The origin of growth depression can be rather an occasional imbalance of amino acids (Skurray, 1973).

This review shows that the production and use of meat-and-bone meals in the feed of livestock is a favourable method for recycling the slaughter by-products. The advantage of this method is, besides the reduction of the problems of environmental pollution caused by slaughter by-products, a more efficient utilization of feed resources.

Acknowledgement

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BIOCHEMICAL AND SEROLOGICAL EXAMINATION OF SOME *MYCOPLASMA* STRAINS OF GOOSE ORIGIN

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(Received June 29, 1984)

Cultural, biochemical and serological characteristics of five *Mycoplasma* strains isolated from the airsac and the phallus lymph of geese were studied. All strains showed morphological characteristics typical of the class Mollicutes. They required cholesterol and were inhibited by digitonin. Strains 46, 1219, 1221 and 1222 were glucose-negative and arginine-positive, strain 1220 splitted glucose, but not arginine. According to the growth-inhibition and immunofluorescence tests strains 46 and 1221 formed a distinct serological group, strains 1219 and 1222 belonged to another, while strain 1220 represented the third group. All strains differed serologically from all known avian mycoplasma reference species as well as 48 mammalian mycoplasma reference strains.

Keywords. *Mycoplasma*, goose, biochemical examination, serological examination.

The chronic respiratory disease characterized by fibrinous airsacculitis and peritonitis was often recognized, besides chicken and turkey, in geese (Vuillaume, 1983). From the lesions members of Mycoplasmatales were isolated (Kosovac and Djurisic, 1970; Stipkovits et al., 1975). Among the isolates, *Mycoplasma gallinarum*, *Acholeplasma laidlawii* and *A. axanthum* (Stipkovits et al., 1975) have been found and many cultures have remained unidentified. As a result of studies on mycoplasma infection of geese in the last years (Stipkovits et al., 1984*a*, *b*), at present several isolates are in our hand.

In this paper the results of biochemical and serological examination of 5 strains of goose origin are presented.

Materials and methods

Media. For isolation and further cultivation of strains, medium B (Ernø and Stipkovits, 1973*a*) and standard mycoplasma medium (Tully and Razin, 1969) were used. For biochemical characterization, various modifications of medium B (Ernø and Stipkovits, 1973*b*) were applied.

Strains. The five strains subjected to characterization tests were strain 46 isolated from the airsac of adult laying geese, and strains 1219, 1220, 1221 and 1222 cultured from the phallus lymph of ganders. All strains were filtered and cloned 3 times by the conventional method, with 450-nm Millipore filters (Subcommittee, 1972).

Examination of the strains for criteria of the order Mycoplasmatales

Morphology. Organisms from 2 to 3 days old fluid cultures were stained with Giemsa solution and examined by light microscopy. Unfixed organisms were examined by dark-field microscopy. The colony morphology was studied by stereomicroscope, and Dienes's stain was used.

Microorganisms were examined by electron microscopy. Two preparative procedures were used: ultra-thin sectioning and negative staining. The fluid cultures were centrifuged. The supernatant fraction was discarded; the sedimented cultures and agar cultures of microorganisms were fixed, first in 5% glutaraldehyde, then in 1% osmium tetroxide, dehydrated in step-graded ethanol, embedded in Durcupan and cut into ultra-thin sections. For negative staining the formvar-coated and carbon-stabilized grids were covered with a small drop of centrifuged microorganism suspension, dried for one minute, excess fluid was drained by touching the edge of the grid with filter paper, and the preparation was stained for 90 s with a drop of 2% phosphotungstic acid (PTA) in distilled water adjusted to pH 6.5–7.0 by adding KOH. The grids were examined and photographed in a JEM JEOL 100S electron microscope.

Filtration studies. The filterability of the strains was checked by using membrane filters of 450 and 220 nm.

Reversion studies. The strains were carried through five consecutive passages in fluid medium B without penicillin and thallium acetate. At the end of experiments the final broth culture was examined by microscopy.

Examination of the strains for criteria of family

Sterol requirement for growth. The strains were passaged in medium B broth without serum. Inhibition of growth by digitonin was examined according to the technique of Freundt et al. (1973). Sterol requirement was determined by the method of Edward (1971) using series of various media.

Examination of the strains for criteria of genus and species

Biochemical tests. The strains were examined for ability to split glucose, arginine and urea in broth, and for their arbutin- and aesculin-hydrolysing capability on agar plates according to the technique described elsewhere (Ernø and Stipkovits, 1973*b*). Phosphatase activity and tetrazolium reduction were investigated according to the method of Aluotto et al. (1970), the film and spot production was checked as described by Fabricant and Freundt (1967). In each test two mycoplasma strains, a positive and a negative, were used for inoculating media with and without substrates for comparison.

Serological tests. The strains were examined by the growth-inhibition (GI) test as described by the Working Groups of WHO/FAO Programme on Compar-

ative Mycoplasmology (1976), using the antisera prepared against the following reference strains: avian strains: *M. anatis* 1340; *M. columbinasale* 694; *M. gallinaceum* DD; *M. gallinarum* PG 16; *M. gallisepticum* X95; *M. gallopavonis* WR1; *M. iners* PG 30; *M. iowae* 695; *M. meleagridis* 17529; *A. laidlawii* PG 8; *A. axanthum* 610; as well as bovine strains: *M. alkalescens* D12; *M. arginini* G 230; *M. bovigenitalium* PG 11; *M. bovirhinis* PG 43; *M. bovis* Donetta; *M. bovoculi* M 165/69; *M. canadense* 257 C; *M. dispar* 462/2; *M. gateae* CS; M. *mycoides* subsp. *mycoides* PG 1; *M. verecundum* ATCC 10145; *A. modicum* PG 49 Squire; swine strains: *M. hyopneumoniae* J; *M. hyorhinis* BTS-7; *M. hyosynoviae* S 16; *A. granularum* Friend; ovine and goat strains: *M. agalactiae* PG 2; *M. capricolum* Calif. Kid; *M. conjunctivae* HRC 581; *M. mycoides* subsp. *capri* PG 3; *M. ovipneumoniae* Y 98; *M. putrefaciens* KS-1; *A. oculusi* 19 L; Type 5 (G145); Type 7 (A13143); Type 11 (2D); human strains; *M. buccale* CH 20247; *M. faucium* DC 333; *M. fermentans* PG 18; *M. hominis* PG 21; *M. lipophilum* MaBy; *M. orale* CH 19299; *M. pneumoniae* FH; *M. salivarium* PG 20; and other strains: *M. arthritidis* PG 6; *M. canis* PG 14; *M. caviae* G 122; *M. edwardii* PG 24; *M. equigenitale* T 37; *M. equirhinis* M 432/72; *M. feliminitum* BEN; *M. felis* CO; *M. maculosum* PG 15; *M. neurolyticum* Sabin A; *M. primatum* HRC 292; *M. pulmonis* PG 34; *M. spumans* PG 13.

Antisera prepared against the investigated goose strains, namely antiserum 376 obtained against strain 46, antiserum 2840 reacting with strain 1219 and antiserum 2846 against strain 1220, were used in comparative tests.

Serological investigation was also performed by indirect immunofluorescence (IF) as described by Del Giudice et al. (1967), but using only antisera prepared against goose strains.

Results

Criteria for the order Mycoplasmatales

Cultural characteristics. All of the strains grew in medium B within 2–4 days at 37 °C aerobically, more intensively in the presence than in the absence of 5% carbon dioxide. All strains formed typical "fried-egg" colonies on solid media, but colonies without central papilla were also seen (Fig. 1). None of the strains grew on blood agar, except strain 1220, which produced tiny (0.5 mm) colonies showing alpha-type haemolysis. No changes of colony size or colony morphology of this strain were observed after 16 passages made from blood agar to blood agar.

Morphological characteristics. All strains were Gram-negative. In Giemsa-stained preparations mainly coccobacillary forms were observed. In dark-field microscopy also coccoid elements with some pleomorphism were detected.

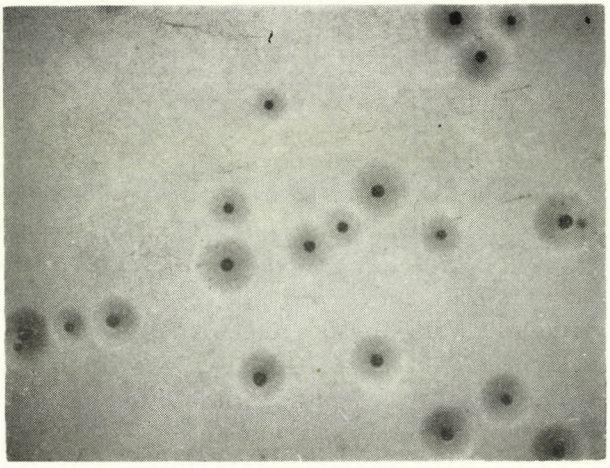

Fig. 1. Colonies of mycoplasma strain 1221 on agar plate, cultured for 72 h and stained with Dienes's stain. ×40

Electron microscope studies of ultra-thin sections revealed pleomorphism (Figs 2 and 3). The cell size varied from 160 to 480 nm for strain 1221 and from 180 to 1700 nm for the other strains. All strains had finely granular cytoplasm, sometimes membrane-bound vacuoles were seen (Figs 4 and 5). In all strains a three-layered compound membrane (unit membrane) was demonstrated. In negatively-stained preparations long branching filaments, small blebs and swollen spherules were found (Figs 6 and 7).

Filtration studies. All strains passed through the 450-nm filter (Table I).

Reversion studies. Based on colony form, cultural characteristics, light-microscopy and dark-field microscopy examination, there was no evidence of

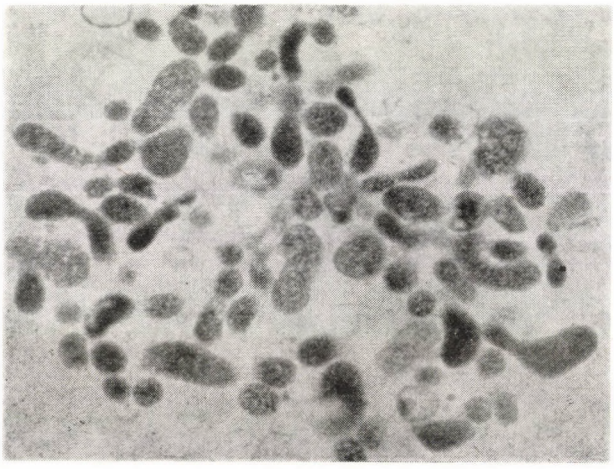

Fig. 2. Pleomorph ultrastructure of 24-h-old agar culture of strain 1222. ×25,000

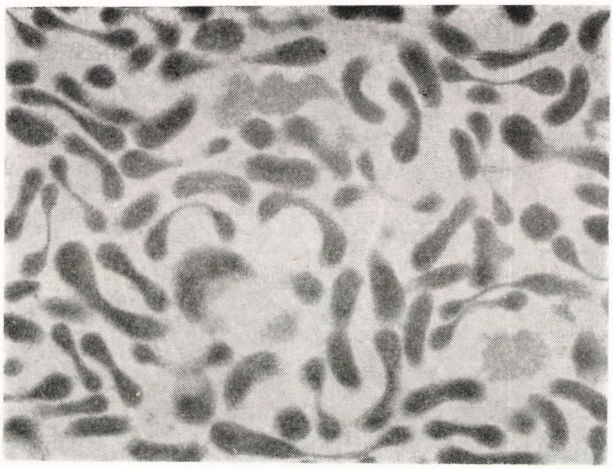

Fig. 3. Pleomorph ultrastructure of 24-h-old fluid culture of strain 1222. ×31,000

Table I

Cultural and biochemical characteristics of goose mycoplasmas

Tests performed		Strains				
		46	1222 8387/3/1/4	1221 8388/7	1219 8389/3/1/4	1220 8390/3/2/1
Filtration through 450 nm	Before	1.6×10^{7}	2.3×10^{8}	2.0×10^{7}	1.2×10^{6}	1.2×10^{8}
	After	1.8×10^{7}	1.5×10^{8}	1.7×10^{7}	1.7×10^{5}	1.9×10^{8}
220 nm	After	3.2×10^{6}	1.8×10^{4}	2.0×10^{7}	4.7×10^{5}	1.0×10^{1}
Glucose-splitting		−	−	−	−	+
Arginine-hydrolysing		+	+	+	+	−
Phosphatase formation		−	−	−	−	+
Digitonin sensitivity		+	+	+	+	+
Cholesterol requirement		+	+	+	+	+
Growth on blood agar		−	−	−	−	+
Haemolysis		−	−	−	−	+

reversion to cell-walled bacteria after five passages in medium B without bacterial inhibitors.

Criteria for family

Sterol requirement. All strains could be cultured for only 2 passages in medium B without horse serum. The washed suspension of cells of the strains did not produce growth on media free from sterol, but grew in media supplemented with 50 μg of cholesterol per ml.

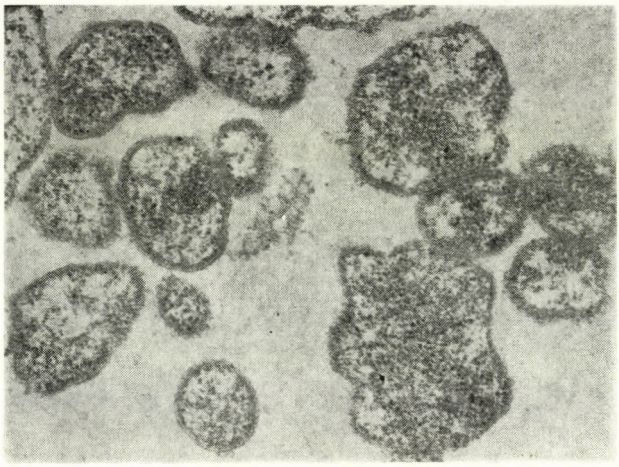

Fig. 4. Ultra-thin section preparation of 24-h-old agar culture of strain 1220. Organisms have finely granular cytoplasm and three-layered compound membrane. ×38,000

Digitonin sensitivity. Growth of all of the strains was inhibited by digitonin-containing disc, producing inhibitory zones 4–5 mm in diameter.

Criteria for genus and species

Biochemical characteristics. All strains were arbutin-, aesculin-, film and spot- and urea-negative. Other biochemical characteristics are given in Table I.

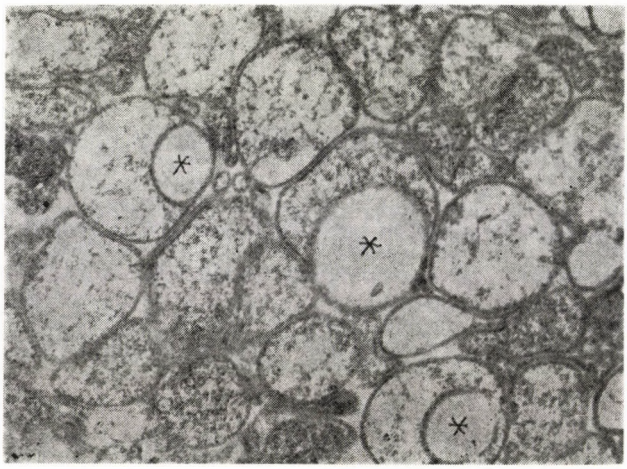

Fig. 5. Ultra-thin section preparation of 24-h-old fluid culture of strain 1219. Organisms have three-layered compound membrane. Sometimes organisms have a membrane-bound vacuole (*). ×19,500

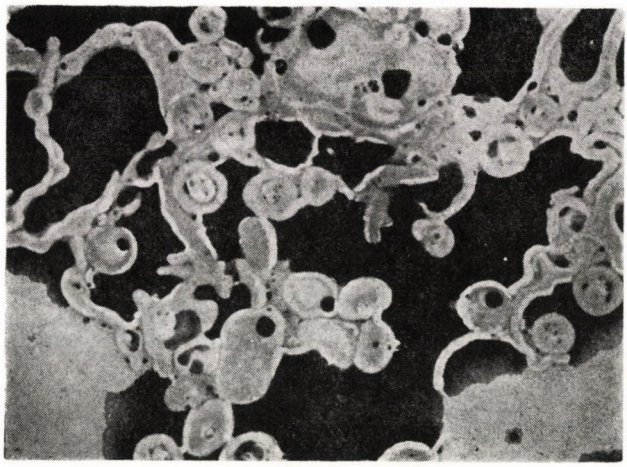

Fig. 6. Ultrastructure of 24-h-old fluid culture of strain 1221. Negative staining. Long filaments are seen. ×15,000

Serological studies. None of the 5 strains under investigation reacted in GI with any of the antisera prepared against known avian and mammalian mycoplasma and acholeplasma species. The strains used for immunization reacted only with homologous antisera (Table II). Strain 1221 reacted with antiserum 376, a serum prepared against strain 46, and strain 1219 proved to be identical with strain 1222.

The results of GI were confirmed by the IF test, namely the strains reacted with homologous antisera but did not with heterologous ones. Strain 1221 reacted with antiserum 376, while strain 1219 with antiserum 2839 (Table II).

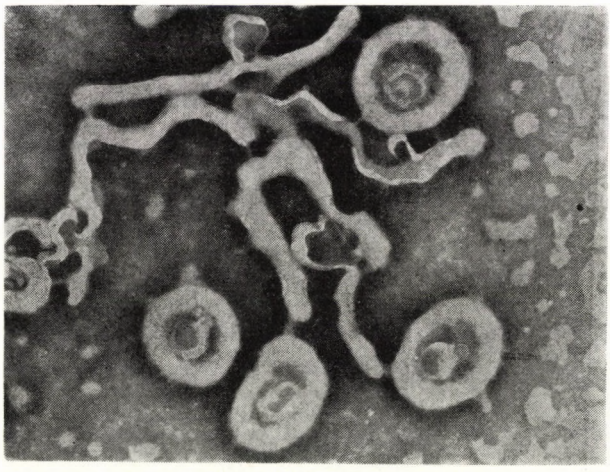

Fig. 7. Electron micrograph of strain 1219 in fluid culture. Note the branching filaments and spherules. Negative staining. ×32,000

Table II

Results of serological tests

Antisera	Strains	46		1219		1220		1221		1222	
		IF	GI	IF	GI	IF	GI	IF	GI	IF	GI
376 (against 46)		+++	4.0	—	—	—	—	+++	3.6	—	—
2844 (against 1220)		—	—	—	—	+++	4.5	—	—	—	—
2839 (against 1222)		—	—	+++	5.1	—	—	—	—	+++	3.0
Antisera against 60 other species		nt	—	nt	—	nt	—	nt	—	nt	—

Remarks: IF = immunofluorescence test;
GI = growth-inhibition test;
nt = not tested

Discussion

All strains probably belong to the class Mollicutes as indicated by the typical fried-egg morphology of the colonies, the pleomorphism of cell form in dark-field examination and Giemsa-stained preparations, the absence of cell wall and the presence of triple-layered membrane in the electron microgram, the absence of reversion to bacterial forms, and passage of cells through 450-nm filters.

The inhibition of growth of all strains by digitonin and their failure to grow in sterol-free and serum-free media might place them in the family Mycoplasmataceae. The inability to utilize urea meets the criteria of their inclusion in the genus *Mycoplasma*.

The serological studies based on GI show that all strains are different from the known species used for comparison. According to the results of GI and IF, the investigated goose strains may be placed in 3 groups: strains 46 and 1221 form the first group, strains 1219 and 1222 the second one, while strain 1220 represents the third group.

Since the antisera used for comparison represented most of the relevant *Mycoplasma* and *Acholeplasma* species required to be tested for taxonomic purposes, the investigated strains seem to represent new *Mycoplasma* species. To confirm this supposition, the DNA base composition of the strains should be determined, *Mycoplasma* species not studied during the present work should be included for comparison, furthermore, some other serological tests (like MI (metabolic inhibition), immunodiffusion) and two-way examinations should be performed for the final characterization of these strains.

Acknowledgements

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Acta Veterinaria Hungarica 32 (3—4), pp. 127—129 (1984)

EFFICACY OF LASALOCID SODIUM AGAINST MYCOPLASMAS (PRELIMINARY COMMUNICATION)

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The so-called ionophore (polyether monocarbonic acid-type) antibiotics represent a new family of anticoccidial compounds. These drugs possess a variety of advantageous properties not owned by the conventional coccidiostatic compounds.

At present three ionophore antibiotics, viz. lasalocid, monensin and salinomycin, are being used in Hungary in broiler chick production to prevent the adverse effects of coccidioses.

During the field trials conducted with lasalocid, first Bozzai (1977), then Udvarhelyi (1979) observed that lasalocid reduced the number of birds succumbed to mycoplasmosis. The present authors re-investigated the above observation in aimed experiments. The results of these preliminary experiments are presented below.

In vitro experiments

Lasalocid sodium (lasalocid-Na) was dissolved in distilled water (50 mg/100 ml) or in ethanol (1 g/100 ml). Of the stock solution, dilutions of 100.0, 50.0, 10.0, 5.0, 1.0, 0.5, 0.1, 0.05 and 0.01 μg/ml concentration were prepared in 1 ml "B" mycoplasma medium. The nutrient medium series obtained in this way was inoculated with broth cultures of *Mycoplasma gallisepticum* (S_6), *M. gallisepticum* (MS-16), *Mycoplasma* sp. (of goose origin), *Acholeplasma laidlawii* (PG 8), *M. hyorhinis* (BTS-7) and *M. bovis* (Donetta), containing 10^3 colony-forming units (cfu). For each microorganism one tube, free of lasalocid sodium, was inoculated and served as control. The tubes were incubated at 37 °C for 3 days. From each tube 0.01 ml nutrient medium was inoculated on agar plates daily. After incubating the agar plates at 37 °C for 4 days in the presence of CO_2, the cfu was determined. With glucose-splitting strains, the change of colour caused by the growth of mycoplasmas and decomposition of the substrate was also monitored (Table I).

Table I

Anti-mycoplasma effect of lasalocid sodium (μg/ml)

Strains	Lasalocid		Tylan	
	mycoplasma-static	mycoplasma-cidal	mycoplasma-static	mycoplasma-cidal
M. gallisepticum S_6	5.0	5.0	0.05	0.05
M. bovis (Donetta)	5.0	10.0	0.5	0.5
M. hyorhinis (BTS-7)	5.0	10.0	1.0	5.0
M. sp. 8389 (of goose origin)	10.0	50.0	0.1	0.1
A. laidlawii (PG 8)	10.0	50.0	0.1	0.1
M. gallisepticum (MS-16)	0.1 0.1*	10.0 0.1*	0.1 N.T.	0.1 N.T.

* after incubation for 4 days; N. T. = not tested

In the in vitro experiment, the anti-mycoplasma effect of lasalocid sodium in a concentration of 0.1 to 10.0 μg/ml proved to be mycoplasmastatic, while in a concentration of 5.0 to 50.0 μg/ml mycoplasmacidal. The survival of *Mycoplasma gallisepticum* (MS-16) was followed for 4 days. In this case the minimum inhibitory concentration was 0.1 μg/ml. The mycoplasmastatic and mycoplasmacidal concentrations of tylosin tartrate (Tylan) used as control varied between 0.1 and 1.0 and 0.1 and 5.0 μg/ml, respectively.

In vivo experiment

Fifty 3 weeks old sexed cockerels free from *M. gallisepticum* were assigned to 5 groups of equal size. Birds of Group 1 were inoculated intraperitoneally with 0.3 ml of a mixture of broth cultures of different *M. gallisepticum* strains (8318, 8400, MS-16). Birds of Group 2 were not inoculated but received a diet containing 75 mg/kg lasalocid-Na for 5 days. Birds of Group 3 were inoculated with the culture of *M. gallisepticum* strains as described above and treated with lasalocid-Na, while those of Group 4 were inoculated and fed a diet containing 100 mg/kg Tylan over a period of 5 days. Cockerels of Group 5 served as uninfected and untreated controls. Treatment was started on the day of inoculation.

The chicks were kept under observation for 3 weeks, and their body mass gain was measured individually and recorded on days 7, 14 and 21 of the experiment. The birds were killed, autopsied and examined for the presence of lesions in the respiratory and abdominal organs. Results of the in vivo experiment are shown in Tables II and III.

Table II

Efficacy of lasalocid sodium in the treatment of chicks infected by *Mycoplasma gallisepticum*

Group	Treatment	Infection		Body mass gain in week of the experiment 1	2	3	Total body mass gain	Lesions
1	—	+	$\bar{x}$	247	310	176	733	7/10
			s	18	29	27	7	
2	lasalocid 75 mg/kg	—	$\bar{x}$	303	325	263	891	
			s	50	13	6	32	0/10
3	lasalocid 75 mg/kg	+	$\bar{x}$	250	332	296	878	
			s	54	15	8	8	3/10
4	Tylan 100 mg/kg	+	$\bar{x}$	323	341	314	978	
			s	16	17	38	14	2/10
5	Control	—	$\bar{x}$	263	309	251	823	
			s	10	8	10	10	0/10

Table III

Comparison of the experimental groups by Student's *t* test

Compared groups	Body mass gain in experimental weeks 1	2	3	Total
Infected and uninfected	$P < 0.05$	N.S.	$P < 0.01$	$P < 0.01$
Infected and lasalocid-treated Infected and Tylan-treated	N.S.	N.S.	N.S.	$P < 0.01$
Uninfected, lasalocid-treated Uninfected, untreated	$P < 0.05$	$P < 0.05$	$P < 0.05$	$P < 0.01$

N. S. = not significant

The results indicate that in the mycoplasma-infected group of chicks fed a diet containing 75 mg/kg lasalocid the number of birds exhibiting gross pathological lesions was lower than in the infected and untreated group.

According to data of the body mass gain, although chicks fed therapeutic doses of Tylan showed stronger protection than those given 75 mg/kg lasalocid-Na, it can be stated that the latter compound also possesses an anti-mycoplasma effect. This effect may have great practical importance. Furthermore, it is known from the literature that lasalocid-Na is not incompatible with tiamutin, while the other two ionophores, monensin and salinomycin, are.

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CORRELATIONS BETWEEN THE TITRES OF NATURAL AND IMMUNE ANTIBODIES TO SHEEP ERYTHROCYTES IN INBRED MOUSE STRAINS

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Genetic regulation of the serum "natural" antibody level has been studied to sheep erythrocytes (SE) in different inbred and H-2 congeneic mouse strains. Natural agglutinins were produced by C57B1/10ScSn and their H-2 congeneic partners while not by the other strains tested.

The capability to produce these "natural agglutinins" was inherited recessively in the F_1 hybrids of the producer and nonproducer strains.

Although the distribution of the "high" and "low" serum agglutinin levels after immunization with SE exhibited similar distribution pattern as the "natural antibodies" among the different inbred and H-2 congeneic strains, the inheritance of the high-responsiveness was found to be dominant in the F_1 hybrids.

Keywords. Natural antibodies, immune antibodies, sheep erythrocytes, mouse strains, inheritance.

Natural antibodies are immunoglobulins which can react with well-defined antigens without any previous contact with the antigen in question. They are present in the body fluids in most of the vertebrates (Jormalainen and Mäkelä, 1971; Andó et al., 1978). Their production is presumably induced by ubiquitous bacteria or they may represent a "basic noise" of resting lymphocytes (Lieberman et al., 1974; Wilson et al., 1972). Clones producing natural antibodies which react with a compound may be the relatives of clones secreting immune antibodies after contact with the same determinant (Jormalainen and Mäkelä, 1971). If this is so, the level of natural antibodies reacting with a compound may predict the ability of an organism to mount high or low levels of antibodies induced by the same determinant.

In a previous study correlations were observed between the genetic control of natural and immune antibody levels to a hapten, oxazolone (Andó et al., 1978). In this work, we extend our studies on a complex, naturally occurring antigen, the sheep erythrocyte (SE).

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Materials and methods

Normal sera were prepared from 2–3 months old female mice (Andó et al., 1978). Immune sera were taken 4 days after an intraperitoneal injection of 4×10^6 SE. The same sheep served as erythrocyte donor throughout.

The antibody titres were determined by haemagglutination of a 1% erythrocyte suspension in "V"-bottomed microtiter plates. For titration of induced antibody levels, immune sera were diluted in phosphate-buffered saline (pH 7.2). The natural antibody containing normal sera were diluted with PBS containing 0.5% PVP-K60. Natural antibodies did not agglutinate SE in PBS without PVP-K60. Antibody titres were expressed as the mean in $\log_2$ units.

In all experiments the *standard error* of the mean (SEM) was calculated. The significance of the differences was calculated by Student's *t* test. Only those differences are discussed where $P < 0.01$ was found.

Results

Different inbred strains showed a consistent picture in respect to their capacity to produce natural agglutinins to SE (Table I). Mice of strain C57BL/10ScSn and their H-2 congeneic partners were producers, whereas all other strains were nonproducers. The ability to produce agglutinating antibodies was inherited recessively in two F_1 hybrids of producer and nonproducer strains (Table II, column "A"). Immune sera of the inbred strains tested revealed a similar strain distribution to the natural antibody levels. Higher response was observed in B10, B10.A, B10.BR and B10.D2 than in the other strains of mice. However, contrary to the natural antibody levels, strains with $H\text{-}2^b$ haplotype exhibited slightly lower antibody responses than their H-2 congeneic partners and the high responsiveness was inherited dominantly (Table II, column "B"). All the sera lost their capacity to agglutinate SE after 2-mercaptoethanol treatment showing that the antibodies were of IgM type (data not shown).

Discussion

The above results and the observations of others (Davidsohn and Stern, 1949; Stern and Davidsohn, 1954) demonstrate that agglutinating antibodies to sheep erythrocytes circulate in the blood of different mouse strains even without previous contact with this antigen. On the other hand, IgM-type antibodies are induced by immunization with a low dose of this antigen. The level of both types of antibodies is under genetic control. There is a strong correlation

Table I

Anti sheep erythrocyte titres in the sera of inbred and H-2 congeneic mouse strains

Strain	H-2 haplotype	Haemaggl. titre–$\log_2(x \pm S\bar{x})$	No. of ab. producers[a] / No. of animals tested	Immune antibody titres
B10	b	2.9 ± 0.21	29/30	4.4 ± 0.25
B10.A	a	2.6 ± 0.28	16/17	5.9 ± 0.33
B10.M	f	3.2 ± 0.34	17/18	N.T.[b]
B10.BR	k	2.8 ± 0.28	21/21	5.7 ± 0.19
B10.D2	d	2.9 ± 0.48	15/17	5.5 ± 0.26
B10.RIII	r	3.6 ± 0.52	9/9	N.T.
B10.A(2R)	h2	3.2 ± 0.36	10/11	N.T.
B10.A(4R)	h4	4.5 ± 0.68	8/8	N.T.
B10.A(5R)	i5	3.8 ± 0.54	9/9	N.T.
B10.AKM	m	2.4 ± 0.34	21/23	N.T.
A.BY	b	0.0 ± 0.00	0/18	2.4 ± 0.23
A/Ph	a	0.0 ± 0.00	0/18	2.8 ± 0.19
A.CA	f	0.0 ± 0.00	0/12	N.T.
A.SW	s	0.0 ± 0.00	0/10	N.T.
C3H/DiSn	k	0.0 ± 0.00	0/18	2.4 ± 0.21
C3H.SW	b	0.0 ± 0.00	0/8	1.7 ± 0.29
CBA	k	0.0 ± 0.00	0/14	0.9 ± 0.36
BALB/C	d	0.0 ± 0.00	0/7	N.T.
AKR	k	0.0 ± 0.00	0/20	N.T.
DBA/2	d	0.0 ± 0.00	0/19	1.0 ± 0.31

[a] normal sera
[b] not tested

Table II

Anti sheep erythrocyte titres in the sera of inbred and F_1 hybrid mice

Animals	H-2 phenotype	A[a]	B[b]
B10	bb	2.90 ± 0.21 (29/30)[c]	4.4 ± 0.25 (14)[d]
B10.D2	dd	2.94 ± 0.48 (15/17)	5.5 ± 0.26 (12)
C3H.SW	bb	0.00 ± 0.00 (0/ 8)	1.7 ± 0.29 (7)
DBA/2	dd	0.00 ± 0.00 (0/19)	1.0 ± 0.31 (7)
(B10 × C3H.SW)F_1	bb	0.00 ± 0.00 (0/ 8)	6.0 ± 0.37 (6)
(B10.D2 × DBA/2)F_1	dd	0.00 ± 0.00 (0/12)	6.0 ± 0.26 (6)

[a] Antibody titres ($\log_2$ units) in the sera of nonimmunized mice ($x \pm S\bar{x}$)
[b] Antibody titres ($\log_2$ units) in the sera of mice immunized with 4×10^6 SE i. p. ($x \pm S\bar{x}$)
[c] Number of antibody producers/Number of animals tested
[d] Number of animals tested

between the anti-sheep erythrocyte titres and the "genetic background" of the mouse strain; C57BL/10ScSn genetic background giving a higher titre than the others. There is no regulatory role of the major histocompatibility complex (MHC) linked immune response (Ir) genes over the natural antibody level, while a weak, but significant, Ir gene effect over the primary IgM response is detectable. These findings are consistent with the previous observation that the natural antibody level to oxazolone is under the influence of non-MHC linked genes and likewise T-cell-independent (Andó et al., 1978). The observation that the strain distribution pattern of the natural and immune antibody levels to SE and to oxazolone (Andó et al., 1978) parallels, shows a similar property of the antibody-producing clones possibly as a result of the common ancestral origin (Wilson et al., 1972).

The reverse inheritance of the natural and antigen-induced antibody levels suggests, on the other hand, that the titres are the final outcomes of different regulatory mechanisms, the latter being H-2 and T-cell dependent, while the former T-cell and H-2 independent.

The low natural antibody level in the F_1 hybrids can be interpreted either by the recessive inheritance of the high natural antibody-producing capacity, or by the dominant inheritance of the tolerance in the F_1 hybrids to crossreacting self-antigens. The dominant inheritance of high primary response could be the result of the activation of immune-response genes of the H-2 complex by a T-cell dependent antigen (Andó and Fachet, 1977; Parrott et al., 1970).

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SEXUAL DIMORPHISM OF LONG BONE GROWTH IN CATTLE

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The greatest lengths of the three long bones constituting the thoracic limb have been analysed, using 73 cattle skeletons of various genotypes in order to detect sexual dimorphism in the front extremity's relative growth rates. Statistically significant results and external evidence of an interspecific comparison (human skeletal data) highlighted the synergism between growth hormones and testosterone, which results in more pronounced growth tendencies in bulls.

Keywords. Cattle growth, long bone proportions, sexual dimorphism, regulation of bone growth, statistical analysis.

For more than a century the identification of animal bones from archaeological sites in Europe has been primarily carried out by researchers with veterinary training (Rütimeyer, 1861).

One of the most exciting aspects of archaeozoological analysis, the reconstruction of individual animals, has also been a major focus of the work. The metric variability of skeletons, however, is rarely discussed in a wider, physiological context.

Applied research in this field is usually exhausted by the calculation of "factors" which serve as handy "rules of thumb" in everyday work. On the other hand, this practical approach is by definition normative and thus tends to overlook a detailed understanding of the variability in growth phenomena in the region of the skeleton under discussion.

In this paper, basic research concerning growth dynamics of the extremity bones in cattle is used to highlight the potential effects of sexual dimorphism which interferes in a variety of ways to form the actual skeletal makeup of the individual at the peak of its postnatal ontogeny.

Materials and methods

Due to the obvious difficulties in organizing large-scale test slaughters aimed only at the collection of long bones, this study relies on the analysis of data from 34 skeletons. As a result of the relative scarcity of such readily available complete specimens in museums and university collections (measure-

ments for this paper came from material in 16 institutions), extensive use has been made of comparative materials published in the literature. Table I shows the sex and breed/form composition of the basic data set compiled for the purposes of this study.

Although the material did not permit detailed examination of ontogenetic sequences (only two calves were included in the sample), the wide genetic variability of skeletons in a sense offered a range of "relative ages", as defined by differential maturation of the breeds under discussion.

Table I

Composition of the material used in the study. Parenthesized numbers indicate data from the literature as cited under "Source". Other sources may be decoded using the alphabetic register of institutions listed with the Acknowledgements

Form or breed	Sex		Total	Source
	Cow	Bull		
Aurochs	2	(3) + 1	6	Lehman, 1949; Matolcsi, 1966; 6; 9; 13
Archaeological specimens	(2) + 1		3	Bökönyi, 1951; 8
Buša/Serbian rural breed	(1) + 2	1	4	Boessneck, 1956; 15
Chillingham cattle	2	1	3	3; 13
Simmenthal/Fleckvieh	(2) + 2	(4)	8	Koch, 1927; Boessneck, 1956; Matolcsi, 1966; 1
Grey Hungarian	(6)	(3) + 1*	10	Bökönyi, 1951; Matolcsi, 1966; 2
Jersey cattle		(1)	1	Matolcsi, 1966
Kostroma crossing	1	(1) + 1	3	Matolcsi, 1966; 1
Dachau Turbary Cow	(1)		1	Duerst, 1904
Swedish Red Poll	2			6; 7
Miscellanea	(6) + 17	(6) + 3	32	Dierich, 1910; Koch, 1927; 1–6; 10–12; 14–17
TOTAL	47	26	73	

* The only castrate in the sample — assigned to the group of males.

As far as the composition of the material is concerned, differences between the degree of sexual dimorphism itself might have been expected when extremely different breeds were compared. This phenomenon may be particularly apparent in modern breeds and seems to correlate with the assimilative type of metabolic constitution. In the sample under discussion here, however, skeletal data from relatively early and primitive cattle have been included. Aside from wild cattle and archaeological specimens, data from the literature also belong to this latter group (Buša, Chillingham, Grey Hungarian, "Turbary

cow"), and even relatively modern breeds are represented mostly by references dated to the first half of this century. The same holds true for the majority of miscellaneous breeds. Skeletal data in this group were predominantly recorded in osteological collections which had been established long before the time of intensive cattle rearing. Thus, only a few individuals from more recent collections (Kostroma crossings from Gödöllő and a young bull from Binghamton, for example) may potentially represent extremes within the sample.

Following the principles of allometric growth, greatest length of each long bone in the front extremity has been compared with the theoretical length of that extremity represented by the sum of humerus, radius, and metacarpus lengths (von den Driesch, 1976). Special methodological considerations in this study may be summarized as follows:

1) Although this analysis has been inspired by the archaeozoological attempts to reconstruct stature, only approximate i.e. minimal withers height could have been estimated on mounted skeletons. The above-mentioned "theoretical extremity length" (TEL) was deemed more accurate, and facilitated the use of data from the literature as well. In addition, the use of summarized bone lengths made the evaluation of individual bone lengths as extremity segments more realistic.
2) A traditional archaeozoological approach, on the other hand, has been retained in the use of the length of individual bones as independent variables (x) in predicting TEL (y) in the regression analysis, which was the main statistical tool applied in this paper.
3) While the principle of allometry determined the methodological framework of this analysis, none of the classical logarithmic transformations (Huxley, 1932) have been used. Considering that the decisive majority of individual skeletons available for study were representatives of the adult age group and belonged to the last phase of growth (Fábián, 1969), it was hoped that they would display relative growth tendencies in the form of linear regression even in the case of natural scaling, and that this assumption would be supported by high coefficients of linear correlation. If this holds true, the introduction of logarithmic transformations would have only made the results unnecessarily abstract and thus more complicated to interpret in traditional terms.
4) Relative growth rates between long bones were calculated to minimize environmental distorsions in the highly heterogeneous sample.

Hypothesis and test implications

Growth and development (together with histodifferentiation) are fundamental aspects of ontogeny. The sex and in part the physiological constitution

of the animal are genetically determined, while environment may either stimulate, or set limitations on, the manifestation of inherited growth potentials. Endocrine functions may be regarded as a dynamic buffering system between external and internal changes, and as such have a definite impact on ontogeny.

In Fig. 1, a synthetic longitudinal bone growth model is put forward in a quantified form. It is based on the ground-laying work by Medawar (1945), Habermehl (1961), Schmid (1972) and Kemény (1974). Changes in specific growth rate $\left(\frac{\text{d log size}}{\text{d time}}\right)$ for each part of the skeleton follow a hyperbolic decline (continuous line). When these tendencies are projected on the logistic growth curve symbolizing sexual maturation (dashed line), intersection points between the two types of functions vary from bone to bone. (It should be pointed out that, while the hyperbolic decline of specific growth rates has generally been recognized, sexual maturation may be symbolized by a peaked curve alternatively.) The older the animal is when epiphyseal fusion of a particular bone takes place (lower, asymptotic phase of the hyperboles symbolizing specific growth rate) the more chance there is that the bone will be influenced by gonadal hormones. Accordingly, the more horizontal the continuous line is to the right of the dashed line, the less probability there is that respective bones will have been modified by sexual maturation. This reasoning is analogous to the observation that certain pathologic deformations are most prominent in those areas where bone growth is most rapid and thus they vary with age (Stamp, 1982).

As is shown by Fig. 1, the relative sequence of epiphyseal fusion in limb bones does not strictly follow the "distal to proximal" direction set as a norm by the Hammondian principles. Considerable individual variability is also apparent.

Following early intensive growth, the formation of later maturing skeletal parts is inevitably subject to modification by gonadal hormones which are also responsible for the development of secondary sexual characteristics of a quantitative nature. It was thus hoped that a regression analysis of metric skeletal data would support the following hypothesis:

Sexual dimorphism in long bone sizes and proportions may be interpreted as a result of interference between prolonged longitudinal growth in certain extremity segments and intensifying exposure to gonadal hormones which are present in an ever increasing amount in the blood through the process of sexual maturation.

Should statistical statements not conform with this tendency at the $P \leq 0.05$ level of probability, the research hypothesis must be rejected in favour of the null hypothesis which assumes no obvious relationship between the formation of the front extremity and the sex of cattle.

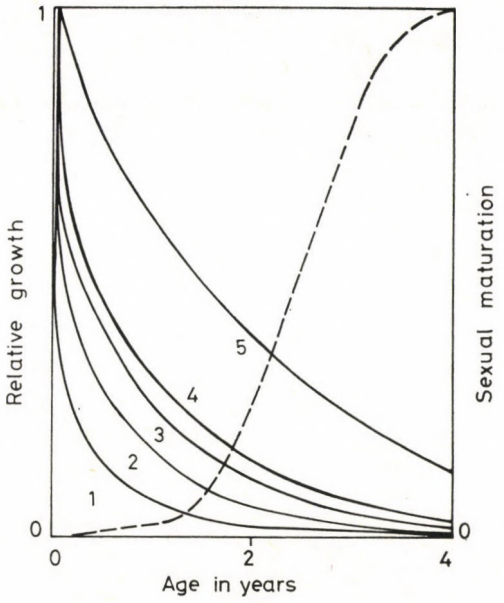

Fig. 1. Schematic representation of the changing relationship between relative growth and sexual maturation (dashed line) during the development of primitive cattle. Differential fusion of the epiphyses is shown by the continuous lines, which read as follows: *1* proximal end of radius, *2* distal end of humerus, *3* distal end of metacarpus, *4* proximal end of humerus and distal end of radius, *5* corpora vertebrae

Results

As an introduction to the problem the theoretical pitfalls in using the so-called "factors" in withers height estimations are pinpointed. According to this approach, withers height is to be obtained by multiplying individual bone lengths (usually from metapodials) by the constant "factor" derived from measurements taken in comparative samples (Boessneck, 1956; Zalkin, 1961; Matolcsi, 1966, etc.).

The underlying assumption is that withers height may be estimated by extrapolation from a constant proportion, rather than from the relationship between actual sizes.

A brief review of Table II suggests that in spite of its attractive practical simplicity this estimation method may be biased by negative and not necessarily high, significant linear correlations between the lengths of bones expressed as the percentage of TEL. While the proportion of the humerus increases consistently at the expense of the metacarpus in both sexes, it is barely correlated with the percentage of radius. The contribution of this latter bone to the extremity as a whole, however, is also negatively correlated with the proportion of metacarpal length.

Table II

Univariate statistics and coefficients of correlation characterizing sex-dependent proportions (%) of the front extremity

	Average percentage and standard error	Coefficient of variation	Coefficient of correlation and standard error	
			radius	metacarpus
Cows n = 47				
humerus	37.283 ± 0.150	0.027	−0.079 ± 0.147	−0.734 ± 0.068
radius	36.698 ± 0.129	0.024	1	−0.618 ± 0.091
metacarpus	26.018 ± 0.190	0.049		1
Bulls n = 26				
humerus	38.602 ± 0.344	0.045	−0.189 ± 0.187	−0.799 ± 0.071
radius	36.354 ± 0.230	0.032	1	−0.439 ± 0.158
metacarpus	25.044 ± 0.376	0.077		1
Total n = 73				
humerus	37.759 ± 0.172	0.039	−0.197 ± 0.172	−0.791 ± 0.044
radius	36.574 ± 0.118	0.027	1	−0.444 ± 0.099
metacarpus	25.666 ± 0.189	0.062		1

Table III

Univariate statistics and coefficients of correlation characterizing sex-dependent relationships between individual bone lengths and TEL. Standard errors of the estimate show the accuracy of predicting TEL from each bone

	Mean value and standard error mm	Coefficient of variation	Coefficient of correlation and standard error	Standard error of the estimate mm
Cows n = 47				
humerus	299.130 ± 6.059	0.137	0.987 ± 0.004	±6.502
radius	294.190 ± 5.696	0.131	0.986 ± 0.004	±6.442
metacarpus	207.651 ± 4.259	0.139	0.951 ± 0.014	±8.897
TEL	800.970 ± 14.811	0.125	—	—
Bulls n = 26				
humerus	342.712 ± 11.035	0.164	0.981 ± 0.008	±11.001
radius	322.020 ± 9.547	0.151	0.979 ± 0.008	± 9.877
metacarpus	219.833 ± 4.762	0.110	0.934 ± 0.025	± 8.694
TEL	884.569 ± 24.617	0.142	—	—
Total n = 73				
humerus	314.870 ± 6.043	0.163	0.983 ± 0.004	± 9.359
radius	304.241 ± 5.294	0.148	0.984 ± 0.004	± 8.102
metacarpus	212.050 ± 2.824	0.113	0.941 ± 0.014	± 8.137
TEL	831.161 ± 13.732	0.140	—	—

Virtual discrepancies of this kind lent an impetus to the analysis of absolute lengths as a means of detecting the possible impact of hormonal effects on sexual dimorphism manifested in long bone growth.

As is shown in Table III, heterogeneity of the material resulted in coefficients of variation exceeding both those in Table II and the norms set for linear traits by Mayr et al. (1953). Coefficients of correlation are with no exception, however, high and significant. This is why it is suggested that linear extrapolation using the regression method should be based on absolute bone lengths in predicting TEL. The standard error of such estimations may be expected to lie within a 1.3—2.2 cm range along the regression line.

Table IV

Regression equations describing relationships between TEL and individual bones

	TEL		Coefficient of integration		Coefficient of regression
Cows n = 47					
humerus	y	=	78.973	+	2.414 x
radius	y	=	46.821	+	2.564 x
metacarpus	y	=	−65.969	+	4.175 x
Bulls n = 26					
humerus	y	=	134.850	+	2.188 x
radius	y	=	71.550	+	2.525 x
metacarpus	y	=	−176.520	+	4.827 x
Total n = 73					
humerus	y	=	127.710	+	2.234 x
radius	y	=	43.887	+	2.588 x
metacarpus	y	=	−138.540	+	4.573 x

Regression equations for each individual bone are listed in Table IV. According to the tabulated coefficients of regression sexual dimorphism is manifested in the growth rate of humerus and metacarpus. In the case of this latter bone 1 mm longitudinal growth in bulls is accompanied by an almost 5 mm increase in TEL. The same increase is 1 mm less in cows. A less marked sexual difference may be seen when the growth intensity of TEL is studied as a function of humerus growth. In this case, however, an opposite tendency may be observed: a 1 mm increase in humerus length of cows is matched by some 2.4 mm growth of the whole extremity, while the same value is only 2.2 mm in the group of bulls. No sexual dimorphism is apparent when coefficients of regression obtained for radius are examined.

Discussion

Statistically significant results verified the use of absolute bone lengths in estimating TEL. The problem central to this paper, however, is to investigate the hypothetical correspondence between quantitative secondary sex characteristics (osteometric data gathered on long bones) and the basic hormonal background of sexual dimorphism.

The control of secondary characteristics in both sexes is the responsibility of gonadal hormones. Androgens are synthesized mostly in the Leydig interstitial cells of the testicles in bulls while oestrogens are produced in the theca folliculi (tunica interna) in the ovaries (Fehér, 1980). The secretion of both hormones is under the influence of the ICSH/LH hormone of the adenohypophysis and intensifies between 6 to 10 months in modern cattle with sexual maturation. The endocrine regulation, however, depends on the integrated action of various hormones and to single out one particular factor would be misleading (Bökönyi and Bartosiewicz, 1983).

According to the widely-accepted heuristic approach, due to their structural and functional characteristics, hormones responsible for the shape of long bones here may be usually sub-divided into two major groups (Sissons, 1971; Kemény, 1974).

The first of these includes the growth hormone produced by the pituitary gland. The major uncertainty has been whether this hormone acts directly on bone-forming cells or affects bone growth by regulating the level of circulating factors (Brand, 1982). It is widely known, however, that excessive amounts of this substance cause abnormal growth of the skeleton. Under experimental circumstances chronic administration of growth hormone resulted in the net increase of bone mass in dogs (Harris and Heaney, 1969).

Growth and sexual maturity depend on many synthetic processes and most of the hormones are involved in their control. The second major methodological group, that of the steroid hormones, may act on the skeleton directly without intervention of other endocrine glands. Of these, testosterone stimulates epiphyseal growth and its influence is synergistic with that of the pituitary growth hormone (Sissons, 1971). Thus gonadectomy in roosters, for example, decreases the intensity of intermediary metabolism causing relative hypothyreosis, which results in a decline in the oxygen absorption of the tissues. On the other hand, increased glycogenesis tends to stimulate protein catabolism, which is also manifested in the less developed musculature and more slender bones in capons (Bökönyi and Bartosiewicz, 1983). The earlier castration takes place, the more marked these effects are. Comparative studies of cattle have shown that steer carcasses had less edible product (muscle) than those of bulls, while gonadectomy of heifers did not have a dramatic effect on carcass characteristics (Klastrup et al., 1984). Oestrogen in general has a retarding effect on bone

growth, while large excess doses of testosterone inhibit growth just as do oestrogens (Silberberg and Silberberg, 1971).

Fundamental work, especially by Hammond (1952) has pointed out that the gross tendency of long bone ossification follows a distal to proximal direction. This observation was also supported by weighing extremity bones and elements from the axial skeleton of calves at various ages.

As shown in Fig. 1, however, bone ossification does not follow this strict sequential pattern. Among the bones under discussion in this study, the metacarpal is unquestionably the first to reach final length. This is shown by the high relative growth rate of TEL resulting from the continuing growth of more proximally located extremity segments. In bulls, the larger coefficient of regression may be explained by the effect of testosterone which reinforces skeletal growth parallelly with sexual maturation as was mentioned in previous paragraphs. This also leads to an accelerated epiphyseal fusion of bull metapodia, while ossification of the metacarpus in heifers is partially completed by the time of the initial secretion of gonadal hormones. The relatively retarded fusion of epiphyseal plates of the humerus makes this bone prone to shaping by sexual dimorphism. As a result, the extremity as a whole maintains a prolonged process of growth relative to its proximal segment (that is the humerus) in females. In other words, the stimulating effect of testosterone ensures that this proximally located bone grows more harmonically with TEL in bulls.

The longitudinal growth of the extremity is remarkably consistent with the change of radius length in both sexes. For this reason the radius is proposed as a good bone for withers height estimations in pooled archaeozoological samples whenever it is preserved in its total length.

Recently, a series of data on live weight and smallest breadth to greatest length proportion of the radius in sixty Dutch-Friesian half sibs have been published by Bergström and Wijngaarden-Bakker (1983). Following the computational schedule of this paper their results were subjected to a regression analysis. Thus the lack of marked sexual dimorphism in the relative growth of radius may be confirmed from another aspect: in spite of the slight difference in the "slenderness" of the bone itself, this proportion (y) changes as the function of live weight (x) in the very same way in both sexes:

$$\text{Males:} \quad y = 15.784 \pm 0.004x \qquad (r = 0.967)$$

$$\text{Females:} \quad y = 15.001 + 0.004x \qquad (r = 0.828)$$

This actual lack of sexual dimorphism in the growth of radius encouraged the complementary use of an interspecific comparison in this study. Physical anthropology has long been concerned with correlations between body dimensions. Traditionally, tabulated series of bone measurements serve the purposes of extrapolation. Breitinger (1938) published such a table for men in which the

greatest length of humerus and that of radius are listed. Another publication (Bach, 1965) summarizes greatest length of humerus and Martin's lb length of radius for women. Ignoring the minor technical difference between the two radius lengths measured, one may reasonably estimate growth rates for radius (x) in humans as was done with the TEL for cattle in Table IV. In this arrangement the following linear equations were obtained:

$$\text{Males:} \quad y = -96.371 + 1.103x \qquad (r = 0.998)$$

$$\text{Females:} \quad y = -53.137 + 0.912x \qquad (r = 1.000)$$

(High coefficients of correlation result from the use of mean values in the tables analysed.) Naturally, metacarpalia could not be taken into consideration in humans and the whole static function of the thoracic limb is different in humans from that of cattle. Still, the growth intensity of humerus exceeds that of the radius in men, while the growth rate of humerus even falls below the criterion for isometric growth in women.

These results confirm findings obtained by this current work and seem to support the interpretation that the humerus reaches a mature size earlier in female cattle than in bulls. Consider the extremity as a whole: a comparative study of the two sexes has shown that the metacarpus reaches its final size in bulls earlier than in cows, while the opposite holds true for humerus length. In other words, metric data taken on cattle skeletons proved useful in illustrating the synergetic interference between growth hormone and testosterone, while such clearcut tendencies are buffered by later skeletal maturation brought about, among others, by oestrogen in female cattle.

Statistically significant results show that one of the secondary sex characteristics in bulls is a more "polarized" growth schedule which falls in line with known growth principles and which may be associated with the endocrinological makeup of this sex.

Applied archaeozoological research should be aware of the bias introduced by this sexual difference into stature estimations.

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1 Agrártudományi Egyetem (University of Agricultural Sciences), Gödöllő, Hungary
2 Állatorvostudományi Egyetem (University of Veterinary Science), Budapest, Hungary
3 British Museum (Museum of Natural History), London
4 Carnegie Museum of Natural History, Pittsburgh, PA, USA
5 Mezőgazdasági Főiskola (Agricultural College), Kaposvár, Hungary
6 Naturhistoriska Museet, Göteborg, Sweden
7 Naturhistoriska Riksmuseet, Stockholm, Sweden
8 Pilisszentkereszt Archaeological Project, Hungary

9 Royal Scottish Museum, Edinburgh, U. K.
10 State University of New York, Binghamton, NY, USA
11 Stockholms Universitet (Osteologiska Forskningslaboratoriet), Solna, Sweden
12 University of Cambridge (Faculty of Medicine), Cambridge, U. K.
13 University of Cambridge (Zoological Museum), Cambridge, U. K.
14 Univerza v Ljubljani, Ljubljana, Jugoslavia
15 Univerzitet u Beogradu, Beograd, Jugoslavia
16 Uppsala Universitet (Zoologisk Institution), Uppsala, Sweden
17 Zoologiska Museum, Oslo, Norway

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PRODUCTION OF T-2 TOXIN AND RELATED TRICHOTHECENES ON DIFFERENT MEDIA

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Fusarium tricinctum was examined for producing T-2 toxin and its metabolites on cereal substrates. Maximum T-2 toxin level (253 mg/kg) was attained on rice supplemented with 10% glucose and incubated at room temperature (1 week), then at low temperature (1 week), and again at room temperature (1 week). After long-term cultivation T-2 toxin was metabolized into HT-2 toxin and T-2 tetraol, but no significant increase of compounds was observed.

Keywords. *Fusarium tricinctum*, T-2 toxin, trichothecenes, toxin production, cereal substrate.

T-2 toxin and related trichothecenes are potent cytotoxic and immunosuppressive mycotoxins produced by some species of *Fusarium* and other fungi (Smalley and Strong, 1974). Trichothecenes were first detected in naturally mouldy field maize in the United States (Bamburg et al., 1968). It was reported that *Fusarium tricinctum* (syn. *F. sporotrichioides*) produced T-2 toxin on maize culture (Szathmáry et al., 1976).

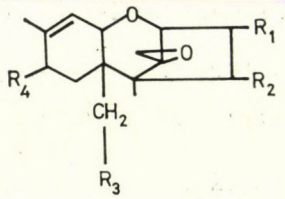
We discovered that T-2 toxin can occur naturally in preharvest field maize (Bata et al., 1983). Trichothecenes can be produced on synthetic media (Kotsonis and Ellison, 1975) and on autoclaved cereal long-term and low-temperature cultures (Burmeister, 1971; Grove et al., 1970; Ueno et al., 1975). Very high quantities of T-2 toxin were obtained when toxic fusaria were cultured on certain, glucose-supplemented media absorbed into vermiculite (Cullen et al., 1982).

This paper reports about the conditions influencing the growing of *F. tricinctum* and its T-2 toxin, HT-2 toxin and T-2 tetraol production (Fig. 1) on cereal substrates and glucose-supplemented media.

Materials and methods

Microorganism

Fusarium tricinctum NRRL 3299 was used in this experiment. Spore inoculum was prepared by growing the fungus on Sabouraud medium at 25 °C for 7 days.



Name	R_1	R_2	R_3	R_4
T-2 toxin	OH	OAc[1]	OAc	OVal[2]
HT-2 toxins	OH	OH	OAc	OVal
Neosolaniol	OH	OAc	OAc	OH
T-2 tetraol toxins	OH	OH	OH	OH

Fig. 1. Some naturally occurring trichothecene toxins (*1* OAc = acetyl, *2* OVal = valeryl)

Media and substrates

The following substrates were studied:

1) Sabouraud agar
2) 74 g grained maize (100 ml) +50 ml water
3) 90 g rice (100 ml) +100 ml water
4) 90 g rice (100 ml) +100 ml of 5, 10 and 20% glucose solution
5) 2 g soybean meal +0.5 g maize steep liquor +100 ml water
6) 2 g soybean meal +0.5 g maize steep liquor +100 ml of 5, 10 and 20% glucose solution.

Culture conditions

Roux bottles containing one of the above-listed substrates were autoclaved for 30 min at 120 °C and inoculated with spore suspension. The temperature and time of incubation are shown in the tables.

Extraction and purification

The content of Roux bottles was extracted with 400 ml of ethyl acetate, then with 400 ml of methanol–water mixture (6:4 v/v). After filtration the two portions were combined and evaporated on a rotary evaporator. 1/10 part was dissolved and placed on a 10 ×1 cm silica gel column. The column was defatted with 30 ml benzene, then the toxins were eluted with 20 ml of benzene–acetone (1:1 v/v). The toxin-containing eluate was divided into two portions for thin-layer chromatographic (TLC) and gas-liquid chromatographic (GLC) analysis and evaporated to dryness (Fig. 2).

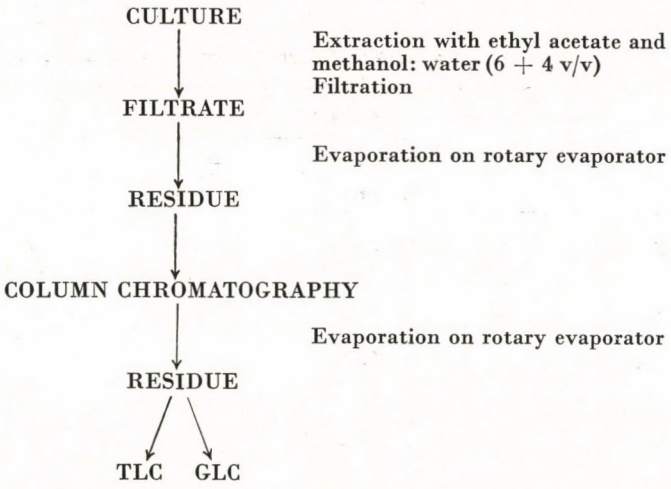


Fig. 2. Extraction and purification of the toxins produced in cultures

Derivatization of the samples was carried out as described previously (Bata et al., 1983).

Gas chromatography

A ten m long glass capillary column (stationary phase SE 52) of 0.30 mm internal diameter (i.d.) was used at a column temperature of 180–260 °C, 4 °C/min.

Thin-layer chromatography

Benzene–acetone mixture (12:7 v/v) was used for developing. The TLC plates were visualized by spraying with 1% 4-(p-nitrobenzyl)pyridine, followed by heating at 150 °C for 30 min and by spraying with 3% tetraethylenepentamine solution (Takitani et al., 1979).

Mycotoxin standards

T-2 toxin obtained from Supelco (4–6322), HT-2 toxin kindly supplied by Dr. M. Palyusik and T-2 tetraol prepared in our laboratory were used.

Results and discussion

Cultivating the fungi at room temperature a time-dependence of trichothecene production can be observed (Table I). The maximum T-2 toxin level was obtained in sample 1 (yielded after 3 weeks of incubation) and it decreased

Table I

Production of trichothecenes at constant temperature

Substrate		Conditions of production		Trichothecenes[a] mg/kg		
		Temperature of incubation	Time of incubation	T-2	HT-2	T-2 tetraol
Maize	1	20–22 °C	3 weeks	52	4	2
	2		4 weeks	18	9	12
	3		5 weeks	Ø[b]	Ø[b]	Ø[b]
Rice	1	20–22 °C	3 weeks	104	18	5
	2		4 weeks	57	16	21
	3		5 weeks	3	Ø[b]	Ø[b]

[a] means of three replications
[b] <0.5 mg/kg

thereafter. GLC determination indicated that sample 2 (gathered after 4 weeks of incubation) contained HT-2 and T-2 tetraol at the highest level. The content of these metabolites also decreased in sample 3 (examined after 5 weeks of incubation).

The effect of the low temperature intervals was studied in the subsequent experiments. As shown in Table II, the maximum yield of T-2 toxin was attained in sample 1 with both substrates. Further variations resulted in the decrease of T-2 toxin and its metabolites and only variation 2 resulted in a moderate increase of the concentration of HT-2 toxin and T-2 tetraol.

Table II

Effect of the low temperature intervals on trichothecene production

Substrate	Sample	Incubated for				Trichothecenes[a]		
		0—7	8—14	15—21	22—28	T-2	HT-2	T-2 tetraol
		days at temperature (°C)				mg/kg		
Maize	1	20–22	5–8	20–22	—	125	15	7
	2	20–22	5–8	20–22	20–22	23	17	21
	3	20–22	5–8	5–8	—	81	5	1
	4	20–22	5–8	5–8	20–22	42	10	17
Rice	1	20–22	5–8	20–22	—	215	24	8
	2	20–22	5–8	5–8	—	80	24	8
	3	20–22	5–8	5–8	—	73	12	3

[a] means of three replications

Based on these preliminary results the following conditions were selected as optimum for T-2 toxin production: rice as substrate; 1 week at 20–22 °C followed by 1 week at 5–8 °C and 1 week at 20–22 °C again as incubation parameters.

The effect of glucose concentration is shown in Table III. The maximum yield of T-2 toxin and metabolites was attained on rice supplemented with about 10% glucose. The carbohydrate-dependence of trichothecene production was also observed when soymeal steep liquor was used as substrate, but the final T-2 toxin concentration was very low.

Table III

Effect of glucose concentration on trichothecene production (incubated for 1 week at 20–22 °C, for 1 week at 5–8 °C and for one week again at 20–22 °C)

Substrate	Trichothecenes		
	T-2	HT-2	T-2 tetraol
	(mg/kg)		
Sabouraud[a]	12	2	Ø[b]
Rice[a]	215	24	8
Rice + 5% glucose[a] solution	237	20	10
Rice + 10% glucose[a] solution	253	21	8
Rice + 20% glucose[a] solution	102	8	2
Soya[a]	14	1	Ø[b]
Soya + 5% glucose[a]	18	2	Ø[b]
Soya + 10% glucose[a]	21	4	1
Soya + 20% glucose[a]	18	2	Ø[b]

[a] means of three replications
[b] <0.5 mg/kg

According to our results, rice supplemented with 10% glucose is an improved, very useful substrate for T-2 production. In addition to its usefulness, the isolation of toxins requires less purification to remove the interfering materials than with the maize substrate.

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OCCURRENCE OF NEW MONOGENEANS OF FAR-EAST ORIGIN ON THE GILLS OF FISHES IN HUNGARY

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In 1983 six monogeneans of Far-East origin, new for Hungary, were demonstrated to occur on the gills of fishes reared in Hungarian pond farms. Of these species, *Dactylogyrus hypophthalmichthys* and *D. scrjabini* were found on the silver carp, *D. aristichthys* on the bighead, *D. sachuensis* on the common carp, while *Pseudodactylogyrus bini* and *P. anguillae* were demonstrated on the eel. This is the first report on the occurrence of *D. sachuensis* in Europe. Earlier the two *Pseudodactylogyrus* species had parasitized only eels living in the Pacific Ocean; however, at present they can be considered to be common parasites of *Anguilla anguilla* at the European coast.

Keywords. Monogenea, *Dactylogyrus*, *Pseudodactylogyrus*, Cyprinidae, *Anguilla*, occurrence, Hungary.

In Hungary, surveys of the Monogenea fauna of fishes were started in the nineteen-sixties. Molnár (1964, 1968, 1976) and Molnár and Németh (1962) described more than 100 monogenean species, among them several new ones, from fishes reared in pond farms and from natural freshwater fishes. These surveys well reflected the monogenean fauna of native European fish species and included some parasites introduced from the Far-East. Of the monogeneans of herbivorous fishes introduced into Hungary in 1963, Molnár (1968) demonstrated only the grasscarp parasites *Dactylogyrus lamellatus* and *D. ctenopharyngodonis*, although also other Far-Eastern species were known to occur in the neighbouring areas of the Soviet Union (Musselius, 1967), the introduction of which species into Hungary was expectable. The first parasite that was not introduced but gained entry into Hungary through natural waters was the common carp parasite *D. achmerowi* (Molnár, 1976); it was followed by the silver carp parasite *D. suchengtaii* (Molnár, 1978).

The present paper reports that by 1983 practically all common carp, grasscarp, bighead and silver carp *Dactylogyrus* spp. naturalized in the European part of the Soviet Union have spread over to Hungary. Furthermore, a *Dactylogyrus* parasite of the common carp, previously known to occur exclusively in Asia, has also been demonstrated. The list of monogeneans newly occurring in Hungary is completed by the two *Pseudodactylogyrus* species parasitizing the gills of eels.

Materials and methods

The test material consisted of parasites collected during an animal health survey of pond farm fishes and common carp used in aquarium experiments.

Dactylogyrus parasites were collected from the gills alive. The parasites were studied in native state or they were placed into ammonium picrate solution or glycerol–gelatine and examined in slide preparations.

Results

(1) In the spring of 1983 dactylogyrosis was observed in silver carp *(Hypophthalmichthys molitrix)* and bighead *(Hypophthalmichthys nobilis)* kept at a high density, under poor management conditions in the wintering and storage ponds of an Eastern Hungarian farm. Since *D. suchengtaii* and *D. nobilis*, the two monogeneans that had already been known to occur on these fishes in Hungary have failed to cause severe infection until now, the present disease outbreak, accompanied by clinical signs, has called our attention, and the detected helminths were subjected to detailed examination. In the silver carp, besides the less frequently occurring *D. suchengtaii* specimens, extremely large numbers of *D. hypophthalmichthys* Achmerow, 1952 were detected from the gill lamellae, while *Dactylogyrus scrjabini* Achmerow, 1954 specimens were found on the gill rakers. In the bighead, besides *D. nobilis* known already previously, most parasite specimens belonged to the species *Dactylogyrus aristichthys* Long et Yu, 1958.

Subsequently the above-mentioned parasites were demonstrated to occur in other Hungarian pond farms as well.

(2) During our aquarium experiments conducted on common carp *(Cyprinus carpio)* fry with other purposes, *Dactylogyrus* infection was diagnosed among the fish kept in aquaria. The fish to be used in the experiment had been transported to the laboratory at 10 days of age. They appeared free from infection for a long time, and parasites could be demonstrated only later. A detailed study of the parasites responsible for the infection revealed the occurrence of a *Dactylogyrus* species previously unknown in Hungary. Later on the species was identified as *Dactylogyrus sachuensis* Ling, 1965. This parasite, so far unknown in Europe, was first collected by Gussev (1955) from Amur wild carp *(Cyprinus carpio haematopterus)* living in the River Amur. At that time only one parasite specimen was available, the data and a drawing of which were given under the name *Dactylogyrus* sp. 1. The species was described in detail by Ling Mo-en (1973), in Chinese language, in a book edited by Chen. After the Amur region and China, the parasite was first described in Hungary, where it produced even fatal dactylogyrosis in common carp under aquarium conditions.

The infection of laboratory-reared stocks by this gill parasite species could be maintained for a long time. However, attempts at collecting *D. sachuensis* specimens from the gills of fish coming directly from pond farms have invariably failed.

(3) Eels *(Anguilla anguilla)* have been introduced into Hungary regularly for about 22 years. Initially the eels were placed out into natural waters, while more recently they are reared in basins under intensive management conditions. Of the monogeneans only *Gyrodactylus anguillae* Ergens, 1960 has been found to occur in this fish species until quite recently. In 1983 we found two other monogenean species, *Pseudodactylogyrus anguillae* (Yin and Proston, 1948) and *P. bini* (Kikuchi, 1929) on the gills of eels kept in intensive cultures. After their first detection, these parasites were demonstrated also in newly-imported stocks and in stocks not placed out yet. The occurrence of *Pseudodactylogyrus* spp. in Europe has already been reported in a short communication (Molnár, 1983).

Discussion

In parasitological surveys conducted in 1983 six *Dactylogyrus* and *Pseudodactylogyrus* species of Far-East origin were demonstrated in fishes reared in Hungary. The appearance in Hungary of the species *Dactylogyrus aristichthys*, *D. hypophthalmichthys* and *D. scrjabini* found on herbivorous fishes had been expected since these parasites had been present on the neighbouring Ukrainian and, presumably, Roumanian territories. The possibility of their gaining entry into Hungary through natural waters was indicated in advance by the appearance of the species *D. achmerowi* and *D. suchengtaii* that had been demonstrated earlier. The fact that these parasites appeared in masses on their specific hosts seems to verify the statement of Musselius (1968) and Bauer et al. (1981), viz. that they may prove to possess considerable pathogenicity. These parasite species have especially great importance in silver carp, since this fish species had earlier been free from *Dactylogyrus* spp. in Hungary, however, it is now exposed to three *Dactylogyrus* species in this country, including *D. suchengtaii* demonstrated in 1978.

The appearance of *D. sachuensis* in Hungary is much more surprising; it was not expected at all. Obviously, in addition to the four *Dactylogyrus* species commonly occurring in Europe *(Dactylogyrus extensus, D. vastator, D. anchoratus* and *D. minutus)*, the Monogenea fauna of the common carp *(Cyprinus carpio)* comprises other, Far-Eastern species *(D. achmerowi, D. falciformis, D. molnari, D. mrazeki* and *D. sachuensis)*. The latter are indigenous parasites of the Amur wild carp and were described by Akhmerov (1952), Gussev (1955), Ergens and Dulmaa (1969), and Ling (1973). It is also known that, during the introduction of the Amur wild carp into Europe, of the above-listed

species *D. achmerowi* reached the European part of the Soviet Union (Gussev, 1962), and then Hungary (Molnár, 1976). Two suppositions exist concerning the appearance of *D. sachuensis*. The more probable one is that this parasite had been introduced to Europe with the Amur wild carp but the latent infection produced by it remained unrecognized. The other possibility is that *D. sachuensis* got into Hungary with the koi carp bred as an aquarium fish.

The demonstration of *Pseudodactylogyrus* spp. is particularly interesting from the biological point of view, since these parasites do not belong to the original parasite fauna of the European eel *(Anguilla anguilla)*. These species were collected by the scientists first describing them from eel species living in the Pacific Ocean. According to Gussev (1965) the species *Anguilla japonica* and *A. reinhardti* can be considered the hosts of these parasites. So far, two authors have reported the appearance of *Pseudodactylogyrus* spp. in European eels. In Japan, Ogawa and Egusa (1976) demonstrated *P. anguillae* and *P. bini*, in the company of the species described by them as *P. microrchis*, on *A. anguilla* kept together with *A. japonica*. In the European part of the Soviet Union Golovin (1977) collected specimens of *P. anguillae* and *P. bini* from *A. anguilla* kept together with *A. japonica* in experimental basins. It would be plausible to suppose that *P. anguillae* and *P. bini* obviously got to Europe with these experimental fish. However, because of the isolation of the above stock and the extensive infection of pigmented eels caught near the coastline of Western Europe, it is more probable that the eels had contracted infection by parasite species which at present can be regarded as their permanent parasites in the vicinity of their original habitat. So far this presumption has been supported by only one author, viz. Welcomme (1981), who reported that in 1975 *Anguilla australis* had been introduced from New Zealand to Italy. It cannot be excluded that other ill-considered fish introductions took place to other European countries as well.

From studies on the pathological significance of the six monogenean species newly demonstrated in Hungary it seems that *D. sachuensis* is the only one that may be disregarded. Presumably this small parasite can produce gill damage similar to that induced by *D. anchoratus* and *D. achmerowi*, but, in spite of its pathogenicity proved by us in aquaria, the losses inflicted by it are far from those caused by *D. vastator* and *D. extensus*.

According to Musselius (1967, 1968), *D. aristichthys* parasitizing the bighead and *D. hypophthalmichthys* living on the silver carp can produce significant gill lesions. Our observations made until now are in agreement with the above finding.

Imada and Muroga (1977) provided detailed data on the pathological significance of the eel parasites *P. bini* and *P. anguillae* (in our opinion the latter species is synonymous with *P. microrchis*). The same authors (Imada and Muroga, 1979) described a method for controlling these parasites under

large-scale conditions. With this knowledge, the preventive and therapeutic treatment of eel stocks reared in Hungary under isolated conditions can give reassuring results.

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FINE STRUCTURE OF THE PROCESS OF OOCYST WALL FORMATION OF *EIMERIA MAXIMA* (APICOMPLEXA: EIMERIINA)

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The fine structure of the macrogamonts and macrogametes of *Eimeria maxima*, a coccidium parasitizing the small intestine of poultry is given. The process of oocyst wall formation was followed by electron micrographs. This was found to be attributed mainly to the activities of wall-forming bodies I and II giving rise to the outer and inner oocyst layers, respectively. Four membranes were also found to participate in the process of oocyst wall formation of *Eimeria maxima*.

Keywords. *Eimeria maxima*, macrogamont, macrogamete, oocyst, wall formation, ultrastructure.

The successful propagation of coccidian parasites is due, at least in part, to the properties of their robust oocysts. Coccidian oocysts withstand adverse environmental conditions and resist certain chemicals. Such oocyst properties are endowed in the oocyst wall. Oocyst wall formation, at least in the Eimeriina, is attributed to the activities of the wall-forming bodies (Scholtyseck and Voigt, 1964; Scholtyseck et al., 1969, 1971). The association of the wall-forming bodies and the parasite membranes, and the role they play in oocyst wall formation is not fully understood. Investigations done on the oocyst wall formation of certain *Eimeria* species continue to shed lights on this complicated process (Varghese, 1975; Wheat et al., 1975; Michael, 1978; Chobotar et al., 1980; Senaud et al., 1980; and Sibert and Speer, 1980).

In the present communication the fine structure of the oocyst wall formation of *Eimeria maxima* is described and presented in photomicrograp hs.

Materials and methods

Thirty White Leghorn chickens, 2–3 weeks old, were inoculated with pure sporulated oocysts of *E. maxima*. The infection dose was 10,000 to 330,000 oocysts. Two chickens were killed at 6-h intervals beginning at 6 h post inoculation (p. i.). Scrapings and small pieces of the middle part of the small intestine were cut, and fixed in 2.5% glutaraldehyde in 2.14% sodium cacodylate buffer of pH 7.4, osmolality of 400, for 2–4 h at 4 °C. The material was then washed in cacodylate buffer and postfixed in 1.5% OsO_4 in the cacodylate buffer

for 2–4 h. These procedures were followed by washing in buffer. The material was dehydrated in grades of ethanol. While in 70% ethanol, the material was stained with 1% uranyl acetate and 1% phosphotungstic acid at 4 °C for 20 h. The material was embedded in araldite or ERL. Sections were cut on an ultramicrotome and examined with a Zeiss EM 9s2 electron microscope.

Results

The young macrogamonts of *E. maxima* were found to be oval in shape, surrounded by a three-membranous pellicle. Wall-forming bodies II appeared first, and they were enclosed in the rough endoplasmic reticulum (ER) cisternae. Wall-forming bodies II were relatively large and were distributed around the nucleus. Wall-forming bodies I were much smaller, not surrounded by membranes, and were in the central part of the parasite. Wall-forming bodies I and II revealed homogeneous osmiophilic contents. The young macrogamonts of *E. maxima* were also characterized by large numbers of large mitochondria arranged at the periphery, and a few amylopectin granules were scattered in the cytoplasm. The nucleus was large and was centrally situated, usually with a prominent nucleolus (Fig. 1).

In the macrogametes of *E. maxima* the three-membranous pellicle disappeared and the macrogametes were surrounded by unit membranes strengthened at parts with ER. Wall-forming bodies I and II had approximately equal diameters. The homogeneously osmiophilic wall-forming bodies I were arranged at the periphery, while wall-forming bodies II showed spongy nature and moved towards the periphery. Large numbers of amylopectin granules of equal size filled the central part of the macrogamete. The peripheral mitochondria were smaller than those of the young macrogamonts. The nucleus was situated in the centre, with a nucleolus (Fig. 2). The young macrogamonts and macrogametes were found in parasitophorous vacuoles rich in intravacuolar tubules.

The process of oocyst wall formation began with changes which took place in the peripherally-arranged wall-forming bodies I. First their contents particulated into small granules. Then the wall-forming bodies disrupted and osmiophilic tiny granules were scattered along the inner side of the zygote unit membrane (Fig. 3, M1). The dispersal of the osmiophilic particles of wall-forming bodies I was found to be irregular. Long canals of the rough ER were seen at the periphery of the zygote. These canals then formed a membrane (Fig. 3, M2) below the scattered osmiophilic particles of wall-forming bodies I.

Changes then took place in wall-forming bodies II. These were spongelike and then particulated and moved to the periphery of the zygote. The contents of wall-forming bodies II were distributed beneath the newly-formed membrane (M2) (Fig. 3) of ER origin. These then coalesced and formed a discontinuous

homogeneous osmiophilic layer around the zygote. In most cases this layer was found to be thicker and more concentrated at the poles of the developing oocyst (Figs 5, 6, 8). The ER canals then formed two additional membranes (Figs 3 and 4, M3, M4) by arrangement underneath the osmiophilic layer formed by wall-forming bodies II. These membranes separated the cytoplasmic contents from the developing outer layer of the oocyst wall. The outermost unit membrane of the macrogamete remained intact and relatively smooth throughout (Figs 3 and 4, M1). Osmiophilic particles were also seen in the parasitophorous vacuole surrounding the zygote, as the particulation of the wall-forming bodies took place at the periphery of the zygote. The process of oocyst wall formation was not possible to follow further, due to fixation difficulties, but the oocyst wall formation of *E. maxima* revealed a relatively thin outer layer followed by a thicker osmiophilic layer (Fig. 7).

Discussion

As seen by light and electron microscopy the oocyst wall of most coccidians is composed of two layers originating from two kinds of cell inclusions. The so-called dark bodies (wall-forming bodies I), which are PAS-positive granules, form the outer layer of the oocyst. The inner oocyst layer is formed by wall-forming bodies II, which correspond to the light-microscopic protein granules (Scholtyseck et al., 1969).

The wall-forming bodies of *E. maxima* appear first as homogeneously osmiophilic in young macrogamonts. As the process of macrogametogenesis proceeds, the contents of wall-forming bodies II change to spongy-like nature. This is in agreement with the general trend seen in the *Eimeria* species reported by other workers (Scholtyseck, 1962; Scholtyseck et al., 1971; Mehlhorn, 1972; Ferguson et al., 1977; Knöbber et al., 1980).

Varghese (1975), studying the zygote and the early oocyst of *E. labbeana*, observed that the first noticeable change in the young zygote was the disaggregation and/or fusion and subsequent disappearance of wall-forming bodies I. Disintegration and gradual dissolution of wall-forming bodies I occasionally happened within vacuoles. The disappearance of wall-forming bodies I coincided with the appearance of an osmiophilic layer beneath the limiting membrane, and this layer became the outer layer of the wall of the future oocyst. Michael (1978) gave similar description of the fate of the wall-forming bodies of *E. acervulina*. Wheat et al. (1975) reported that wall-forming bodies I of *E. mivati* macrogametes became less homogeneous and broke up into small particles which appeared to be distributed into small vesicles. More obvious changes in wall-forming bodies I in the macrogametes of *E. acervulina* were described by Ferguson et al. (1977). They first dissolved and then passed through a transition-

al granular phase which was then replaced by electron-pale material. Chobotar et al. (1980) gave an account of the changes which took place in wall-forming bodies of *E. papillata*, leading to the formation of the oocyst wall. Senaud et al. (1980) described the formation of the oocyst wall of *E. acervulina*. The process of oocyst wall formation of *E. maxima* observed in the present study was essentially similar to the formation of the oocyst wall in other *Eimeria* species. The fine-structural studies on the formation of the oocyst wall have demonstrated the role played by the wall-forming bodies. Accordingly, the oocyst wall is formed of two layers: an outer electron-dense and relatively homogeneous layer, and an inner electron-pale layer (Scholtyseck, 1962; Scholtyseck and Voigt, 1964; Scholtyseck et al., 1969; Scholtyseck et al., 1971; Doens-Juteau and Senaud, 1974; Varghese, 1975; Ferguson et al., 1977; Michael, 1978; Marchiondo et al., 1978; Chobotar et al., 1980; Senaud et al., 1980; Sibert and Speer, 1980).

In *E. maxima*, the species studied here, the outer layer of the oocyst wall was electron-pale and thinner than the electron-dense inner layer.

The number of membranes which are incorporated in the oocyst wall formation in the Eimeriina is varied. Nine membranes were reported in the oocyst wall of *Isospora canaria* and 3 membranes in *I. serini* (Speer and Duszynski, 1975). Four membranes were reported to be fused in the osmiophilic outer and electron-lucent layer of the oocyst wall of *Sarcocystis* spp. In *E. perforans* 5 unit membranes were observed in the oocyst wall (Scholtyseck et al., 1971). Sibert and Speer (1980) observed a total of 5 membranes before and during the oocyst wall formation of *E. nieschulzi*. A single membrane was reported in *E. larimerensis* and in *E. callospermophili* (Roberts et al., 1970). Five membranes were incorporated in the formation of the oocyst wall of *E. papillata* (Chobotar et al., 1980). Senaud et al. (1980) described 3 membranes in the formation of the oocyst wall of *E. acervulina*. In the present study 4 membranes were observed to participate in the formation of the oocyst wall of *E. maxima*. The presence of highly osmiophilic particles in the parasitophorous vacuole surrounding the macrogametes of *E. maxima* and the role they might play in oocyst wall formation need further investigations. Wheat et al. (1975) observed occasionally a dark mass of material, the crescent body, at one end of the parasitophorous vacuole surrounding the macrogamete of *E. mivati*.

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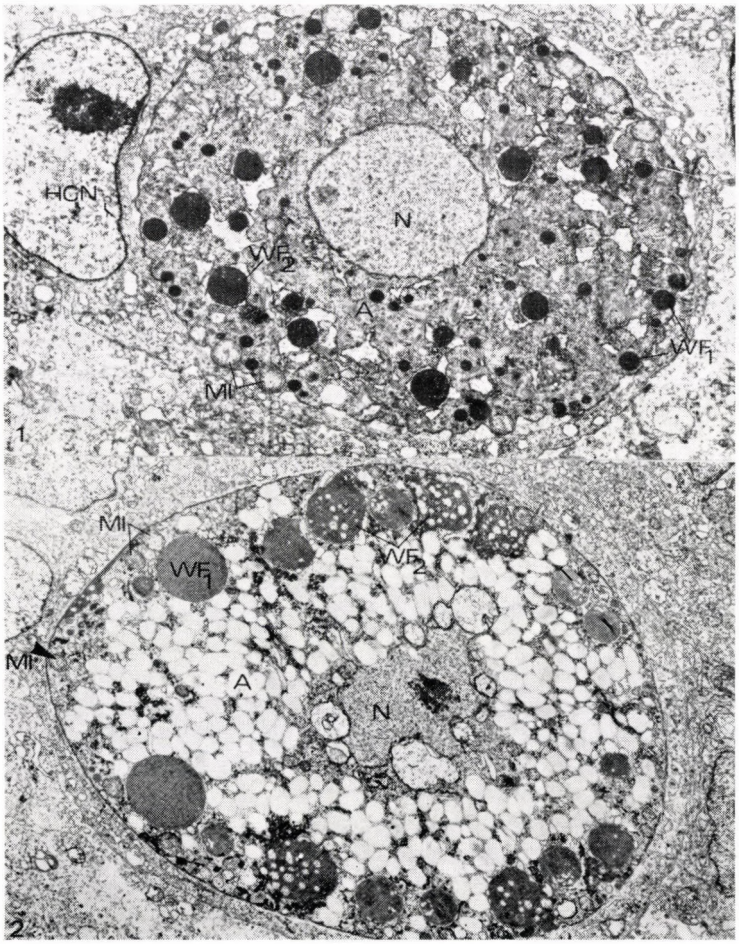
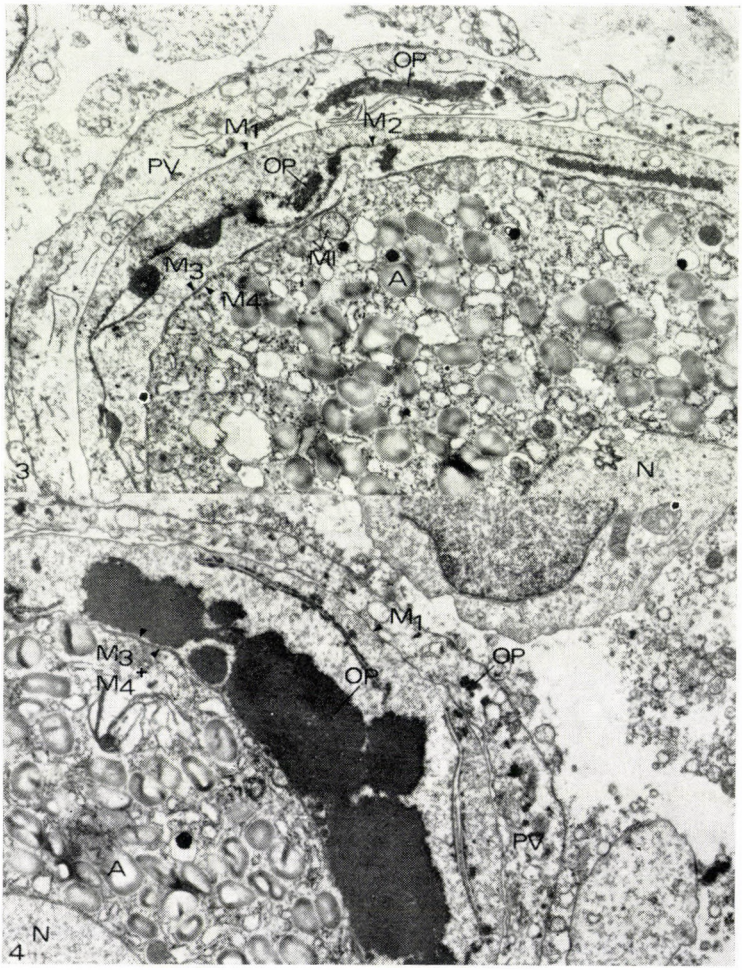


Fig. 1. Eimeria maxima 4–5 days post-infection (p. i.). Electron micrograph of a young macrogamont showing the arrangement of the wall-forming bodies (WF1 and WF2) and the peripheral mitochondria (MI). ×13,500

Fig. 2. E. maxima 5–6 days p. i. Electron micrograph of a macrogamete. Notice the arrangement of the wall-forming bodies and the large numbers of amylopectin (A). ×8850



Figs 3 and 4. E. maxima 5–6 days p. i. Electron micrograph of the oocyst wall formation. Notice the appearance of the osmiophilic particles (OP) in the parasitophorous vacuole (PV). ×30,000

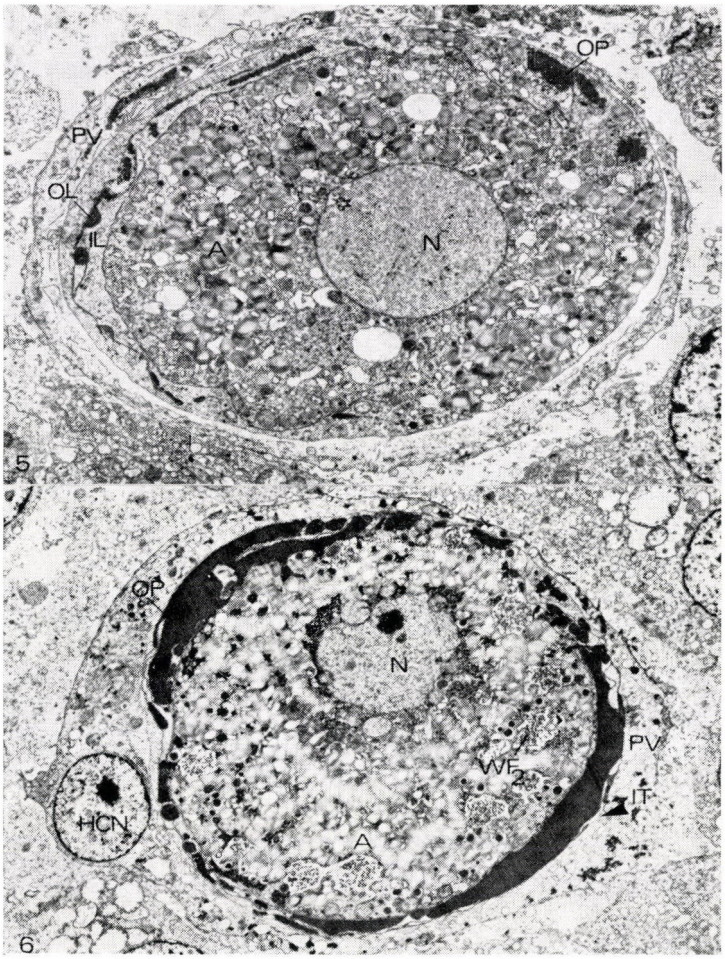


Fig. 5. E. maxima 6–7 days p. i. Electron micrograph of a developing oocyst; regular oocyst wall formation. ×7000

Fig. 6. E. maxima 6–7 days p. i. Electron micrograph of an oocyst within a host cell; irregular oocyst wall formation. ×5700

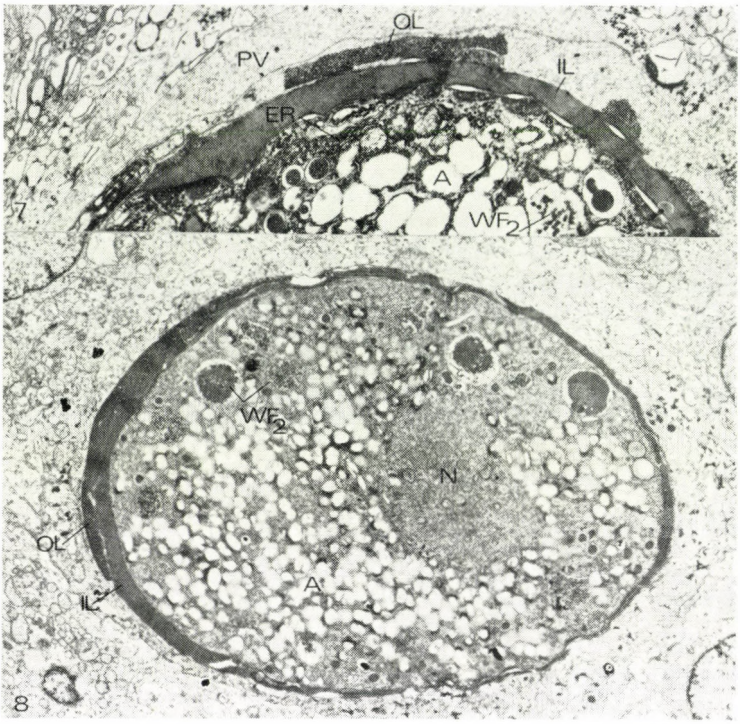


Fig. 7. E. maxima 6–7 days p. i. Electron micrograph of the oocyst wall. ×16,240

Fig. 8. E. maxima 6–7 days p. i. Electron micrograph of a developing oocyst. Notice the particulation of the remaining wall-forming bodies II (WF2). ×8250

Abbreviations: A = amylopectin; ER = endoplasmic reticulum; HCN = host cell nucleus; IL = inner layer of the oocyst wall; IT = intravacuolar tubules; M1–M4 = membranes participating in the oocyst wall formation; MI = mitochondria; N = nucleus; PV = parasitophorous vacuole; OL = outer layer of the oocyst wall; OP = osmiophilic particles; WF1 = wall-forming body I; WF2 = wall-forming body II

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EFFECT OF pH AND TEMPERATURE ON FOUR DEHYDROGENASE ENZYMES OF *HAEMONCHUS CONTORTUS*

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Succinate dehydrogenase (SDH), nicotineamide adenine dinucleotide dehydrogenase (NADH-DH), glucose-6-phosphate dehydrogenase (GPD) and glutamate dehydrogenase (GDH) activities were investigated in adult specimens of *Haemonchus contortus*. Effect of pH and temperature on the activity of these enzymes was studied. SDH exhibited maximum activity (about 120 nmoles/min/mg protein) at an optimum pH of 8.2 and temperature 32 °C, and NADH-DH (about 46 nmoles/min/mg protein) at an optimum pH of 9.0 and temperature 32 and 42 °C. pH and temperature optima for GPD and GDH were 7.4 and 7.0, and 37 °C and 27 °C, respectively. Maximum activity recorded was about 4 and 162 nmoles/min/mg protein, respectively, for GPD and GDH. Possible role of these enzymes in the metabolism of the parasite is discussed.

Keywords. *Haemonchus contortus*, succinate dehydrogenase, nicotineamide adenine dinucleotide dehydrogenase, glucose-6-phosphate dehydrogenase, glutamate dehydrogenase, pH, temperature.

Haemonchus contortus (Nematoda: Trichostrongylidae) is one of the most pathogenic species parasitizing the abomasum of ruminants. It has attracted considerable attention by investigators in different parts of the world (see Sood and Kapur, 1982). Enzymes of different sources are known by one name, even though they may have properties clearly indicating chemical differences. These differentiating characters include pH and temperature optimum values, immunological differences, etc. These differences can be of considerable importance for the chemotherapy of parasitic nematodes. In spite of this, only little attempt has been made in this regard (Barrett and Fairbairn, 1971).

The present studies on *H. contortus* (Rud., 1803) were undertaken to study the effect of pH and temperature on four key dehydrogenases involved in the metabolism of *H. contortus*.

Materials and methods

Adults of *H. contortus* of both sexes were recovered from the abomasum of goats *(Capra hircus)* procured from local abattoirs. The worms were thoroughly washed in saline to remove the adhering materials.

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Worms were homogenized in 0.25 M sucrose for 5 min and centrifuged for 2 min at 2000 rpm. The supernatant thus obtained was employed for assaying enzymes.

Succinate dehydrogenase (SDH) EC 1.3.99.1 was estimated by the method of Arrigoni and Singer (1962), nicotineamide adenine dinucleotide dehydrogenase (NADH-DH) EC 1.6.99.3, glucose-6-phosphate dehydrogenase (GPD) EC 1.1.1.49 and glutamate dehydrogenase (GDH) EC 1.4.1.2 by the methods cited in Bergmeyer's book (1974). Change in extinction was observed at 30 sec intervals for 3 min at 340 nm in the case of GPD and GDH, at 600 nm in the case of SDH and at 420 nm in the case of NADH-DH. Protein was measured by the method of Lowry et al. (1951).

For studying the effect of pH, 0.5 M phosphate buffer was used in the case of SDH and 0.05 M tris-HCl buffer in the case of NADH-DH, GPD and GDH, in the pH range from 6.2 to 9.4. The effect of temperature was studied at the pH of maximum activity, in the range from 22 to 52 °C.

Results

Results of the analysis concerning the four enzymes are given in Table I and Figs 1–3.

With the increase in pH, there was a continuous increase in the SDH activity (Fig. 1) up to pH 8.2, where maximum activity was obtained. This was

Table I

pH and temperature optima of dehydrogenases of *H. contortus*

Enzyme	Optimum pH	Optimum temperature (°C)
SDH	8.2	32
NADH-DH	9.0	32, 42
GPD	7.4	37
GDH	7.0	27

followed by a continuous decrease. With the increase in temperature there was a progressive increase in the enzyme activity till the optimum at 32 °C; thereafter, the activity decreased continuously.

There was a progressive increase in the NADH-DH activity (Fig. 2), with increasing pH; the optimum was reached at pH 9.0. Thereafter, the activity decreased. With regard to the effect of temperature, no particular trend was evident. Optimum values were obtained at 32 °C and 42 °C.

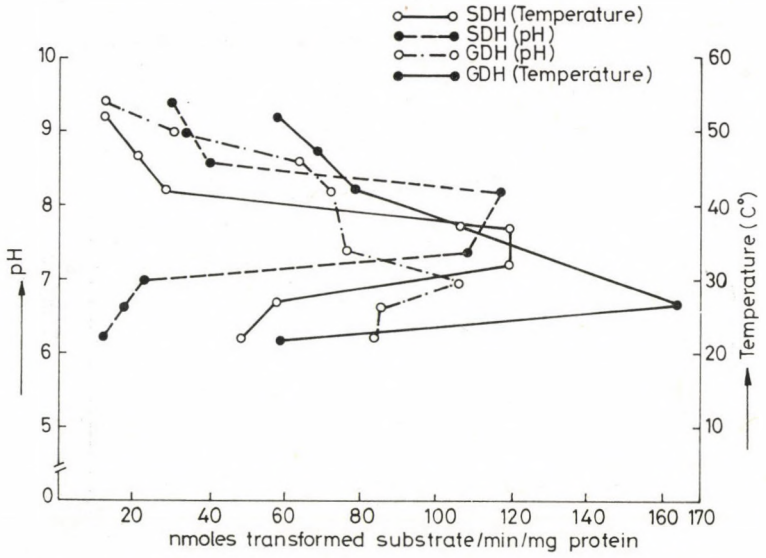

Fig. 1. Effect of pH and temperature on activity of SDH and GDH

Activity of GPD (Fig. 3) increased with increasing pH; optimum was reached at 7.4. This was followed by a continuous decrease; no activity was recorded at 9.4. Similarly, with increasing temperature, there was a progressive

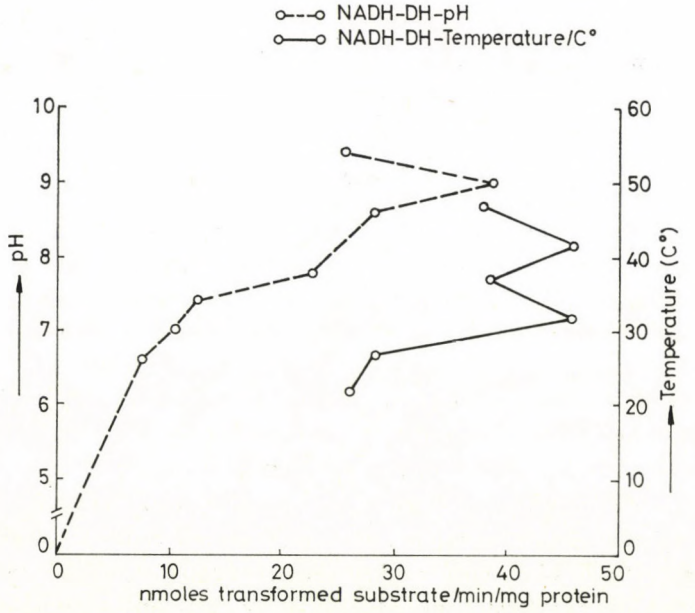

Fig. 2. Effect of pH and temperature on activity of NADH-DH

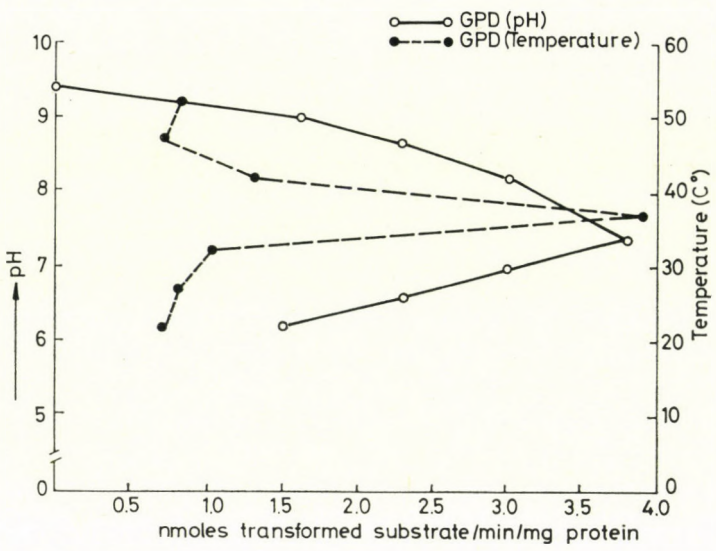

Fig. 3. Effect of pH and temperature on activity of GPD

increase in activity, with the optimum at 37 °C. This was followed by a continuous decrease till 52 °C, where there was a slight increase.

With increase in pH, there was a progressive increase in the activity of GDH (Fig. 1), with optimum at 7.0. Thereafter, the activity dropped rapidly and again increased at 9.0. At pH 9.4, hardly any activity was observed. With regard to the effect of temperature, maximum activity was demonstrated at 27 °C, beyond which, with both increase and decrease in temperature, a continuous decrease in the activity was observed.

Discussion

SDH catalyses the oxidation of succinate to fumarate. The enzyme in *Ancylostoma caninum* has its optimum pH at 9.0 in HCO_3^- buffer and at pH 8.0 in phosphate buffer (Warren, 1965). In *H. contortus* the optimum pH is 8.2 in phosphate buffer, thus being in close agreement with the reported pH optimum for the SDH of *A. caninum*. This indicates that phosphate and HCO_3^- ions have some differential effect on SDH activity in *A. caninum*. In *H. contortus*, the effect of different buffers has not been studied.

NADH-DH is a member of the respiratory chain, acting as a carrier of electrons between NADH and more electropositive components (Harper et al., 1977). Among nematodes, so far it has been detected only in *Ascaris* (Kmetec and Bueding, 1961; Kunimoto and Nagakura, 1974). In *H. contortus*, it is being reported for the first time. It exhibits significant activity at a highly alkaline pH.

GPD, one of the key enzymes of the HMP pathway, has been reported in several species (Anwar et al., 1977; Hermoso et al., 1982). However, there is no evidence that the pathway is involved in energy metabolism in parasitic nematodes. Its main function is probably to provide NADPH and C_5, C_7 sugars for synthetic processes (Barrett, 1976). Its activity in *H. contortus* is nearly 50 percent of that reported in *Ascaris* (Barrett and Beis, 1973), thus indicating its role mainly in synthetic processes; nucleic acid biosynthesis from glucose has been reported by Kapur and Sood (1984). Further, its activity is significantly lower than that of rat liver, in which only 10% of glucose breakdown is by the pentose pathway.

GDH plays a central role in amino acid deamination and in the formation of α-amino nitrogen groups from ammonia. It has been detected only in a few species, viz., *Ascaris*, *A. caninum* and *Dirofilaria immitis* (Perez et al., 1967; Rasero et al., 1968; Langer and Jiampermpoon, 1970; McNeill and Hutchison, 1971). To our knowledge, in *H. contortus* GDH is being reported for the first time. Its activity is very high in comparison to that in *D. immitis* (McNeill and Hutchison, 1971). However, it is very low relative to that in *Fasciola hepatica* (Prichard and Schofield, 1968). This indicates the relatively low importance of α-ketoglutarate dehydrogenase in *H. contortus*.

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COMEDOCARCINOMA AS SYMMETRICAL CUIRASS CANCER IN THE MAMMARY GLANDS OF A FEMALE BOXER

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A case of a bilateral, symmetric comedocarcinoma in the mammary glands of a female boxer is described. The carcinoma formed as cuirass cancer developed metastases in both lymphatic nodes and in the lungs and was analogous to comedocarcinomata of man and cat. A connection between repeated treatment of the dog with gestagene (chlormadinone acetate) and the comedocarcinoma formed as cuirass cancer could be neither confirmed nor refuted.

Keywords. Comedocarcinoma, cuirass cancer, mammary gland, dog, boxer.

In the literature there are only few descriptions of animal comedocarcinomata and cuirass cancers. Up to now, the coincidence of both forms in the same animal has never been observed. The term comedocarcinoma was coined by Bloodgood (1934). At the end of the past century he created it for carcinoma forms of the female mammary glands which grew in the lactiferous ducts and formed a central necrotic cylinder. Upon pressure on the surface it can be squeezed out just like a comedoic clot. On the contrary, a cuirass cancer is a carcinoma form which penetrates the whole mammary gland like a cuirass and which, sometimes, exulcerates.

Diagnosis

Case history and clinical diagnosis

A nine-year old female boxer dog, between May 1976 and July 1981 treated altogether 16 times with chlormadinone acetate in order to suppress heat, was presented at the Clinic of Obstetrics, Gynaecology and Andrology. Clinically, an entirely symmetric tumour could be observed in both mammary glands reaching from the first abdominal teat to the inguinal teats and separated like a groove in the median line. Because of difficulties with respiration and bad general condition, the dog was put to sleep.

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Pathological-anatomical diagnosis

At the dissection an entirely symmetric tumour was found on both mammary glands (Fig. 1); in the area of the second abdominal teat it measured 1 ×3 cm in cross-cut (Figs 2, 3 and 4) and had a solid consistency. The tumour had grown over the two abdominal teats into the inguinal teats (Fig. 5), where it reached a diameter of 4 cm on both sides and extended between the skin and the musculi recti abdominis just like a cuirass in the form of a cylinder. Both inguinal lymphatic nodes were enlarged and dull and had on the cut surface greasy, round foci as large as a grain of sand. Upon pressure, numerous necrotic cylinders could be squeezed out on the cut surface, which were typical and pathognomic for comedocarcinomata. The lungs which, due to the euthanasia, were considerably congested, were clearly hardened and showed small metastases being greasy on the cut surface and resembling the foci of the inguinal lymphatic nodes. The metastases were found in the pulmonary parenchyma in a disseminated form, most frequently in subpleural position. Little stringy mucus indicated the presence of a bronchitis.

Histopathological diagnosis

The diagnoses made on both mammary glands were entirely identical and showed the typical characteristics of a centrally necrotizing comedocarcinoma which in no way can be differentiated from comedocarcinomata in man and also those in the domestic cat. The great polygonal cells (Fig. 6) were arranged concentrically in the duct system of the lacteal gland. Towards the centre, they showed regressive changes up to a central necrosis. Besides considerable displacements of the nucleus–plasma relation, several mitoses occurred as expression of a high growth potential.

The structure of the carcinoma was maintained in both inguinal lymphatic nodes (Fig. 7) as well as in the lung metastases (Fig. 8). Other derivative tumours, in particular the osteometastases occurring often in context with human mammary cancer, could not be demonstrated.

Discussion

Comedocarcinomata occur in man, dog, and cat. With a percentage of 5–6% of the malignant epithelial tumours of the mammary gland, the dog shows a similar frequency to man (Schönbauer, 1981). This carcinoma is of particular interest for comparative pathology; due to the fact that the majority of canine mammary tumours has to be attributed to the mixed mammary tumours, the greatest part of them cannot be taken for comparative studies,

because, apart from very rare carcinosarcomas, mixed tumours in particular, benign tumours do not occur in man. Because of the extraordinary morphological similarity of the comedocarcinoma from which man and dog are suffering, such cases could be taken in order to work out treatment strategies. It is proven that this type of tumour in the dog is malignant (Schönbauer, 1981) and, thus, it shows no difference from human tumours of this kind neither in its biological features. The formation of a unilateral cuirass cancer can sometimes be observed in dogs, particularly in the case of mixed mammary tumours. The growth on both sides which, in addition, is entirely symmetric, constitutes an unprecedented case among our patients which we could not yet prove for comedocarcinomata. According to Bässler (1978), such cuirass cancers as comedocarcinoma occur very seldom in man, a condition which points out the comparative pathological aspect.

As at least a connection between proliferations of the mammary gland and the chlormadinone acetate is already proven (Nelson et al., 1973), we assume a very sceptic attitude towards this method of heat postponement for female dogs, even if in this case a direct connection between the incidence of the carcinoma and the heat postponement by means of chlormadinone acetate could be neither confirmed nor refuted.

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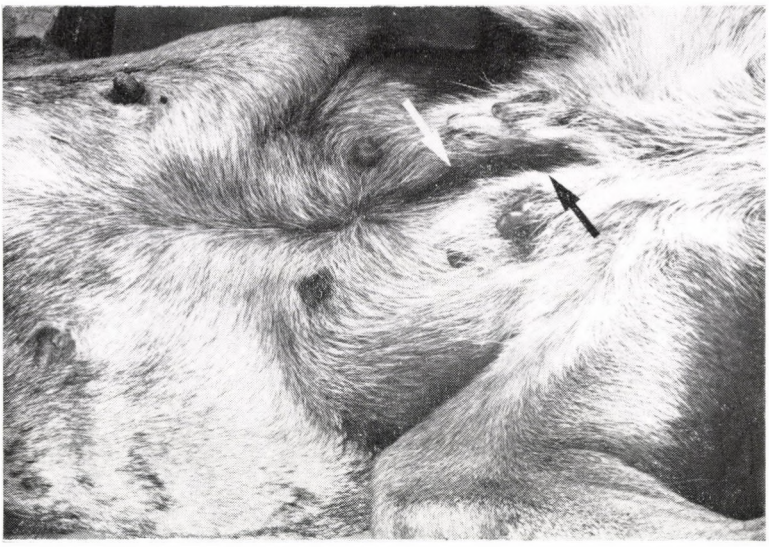

Fig. 1. The carcinoma has entirely grown into the mammary complexes of the dog, starting from the first abdominal teat and continuing caudally. In the median line, they are separated by a groove (arrow)

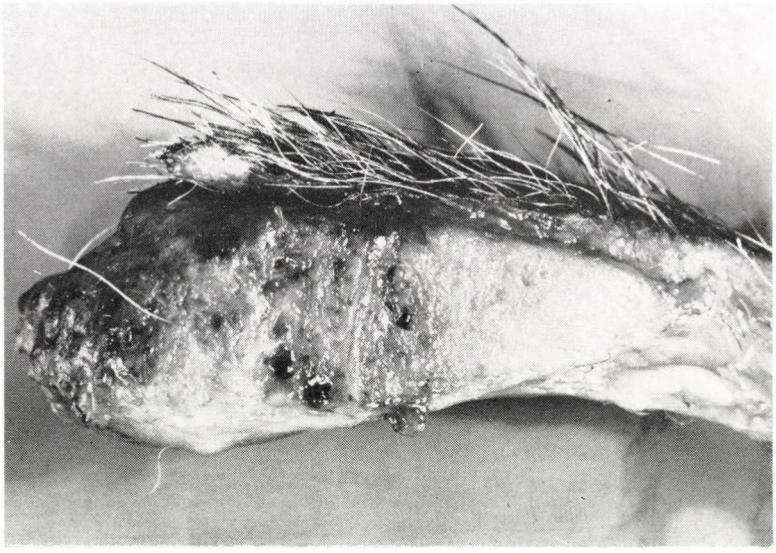

Fig. 2. Like a plate, the tumour lies between skin and muscles. Cut surface between first and second abdominal teat, righthand

Fig. 3. Upon pressure, numerous necrotic cylinders can be squeezed out; cut surface between first and second abdominal teat, righthand. The same area as shown in Fig. 2

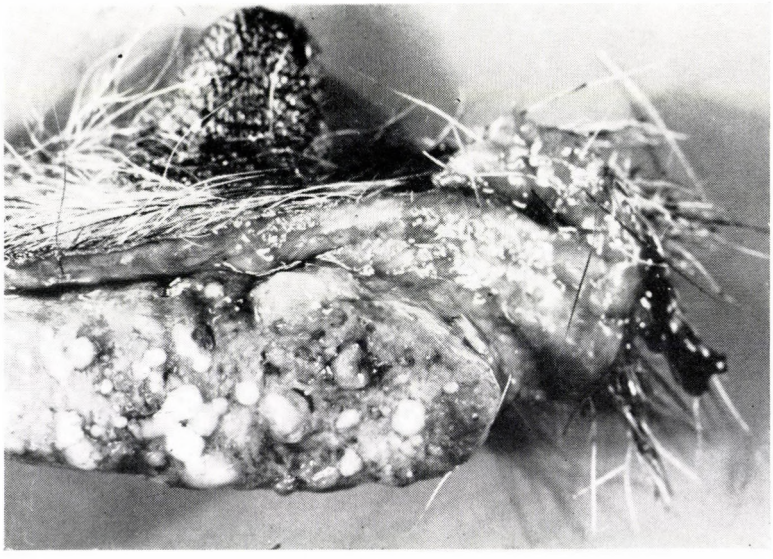

Fig. 4. The left mammary complex is entirely identical, also here necrotic cylinders can be squeezed out; second abdominal teat, lefthand

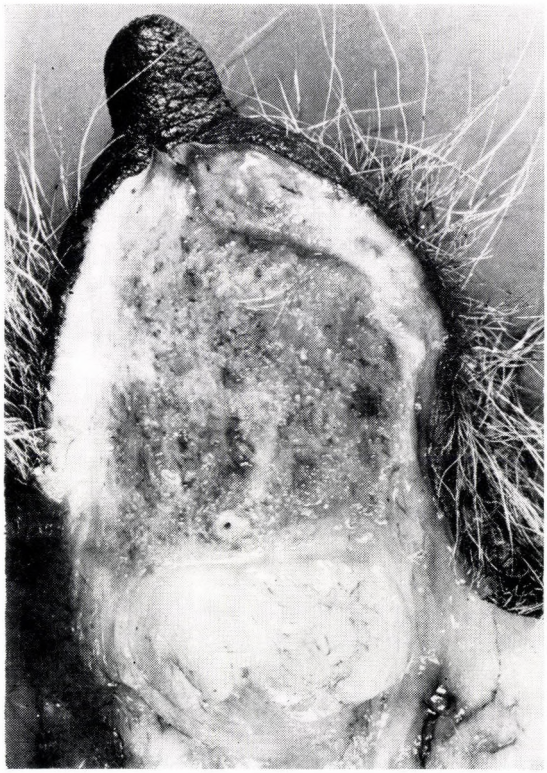

Fig. 5. The carcinoma has enlarged and penetrated into the lacteal gland cylindrically; cut surface at the right inguinal teat

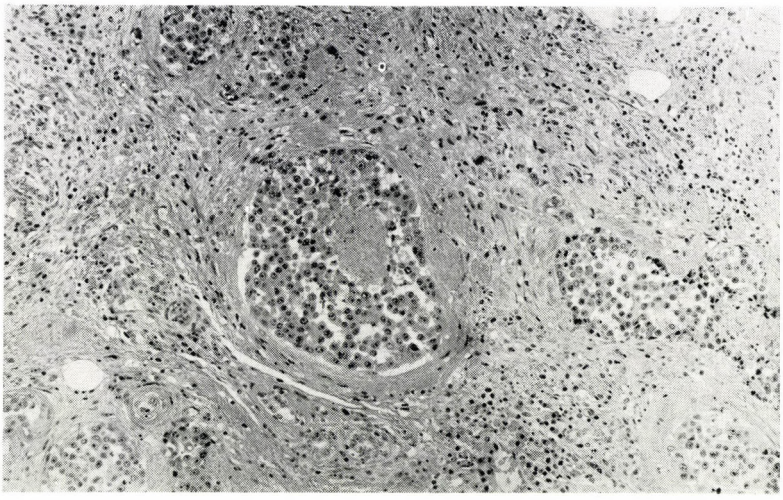

Fig. 6. Comedocarcinoma with central necrosis. Metacrylate-embedded, H. and E. ×250

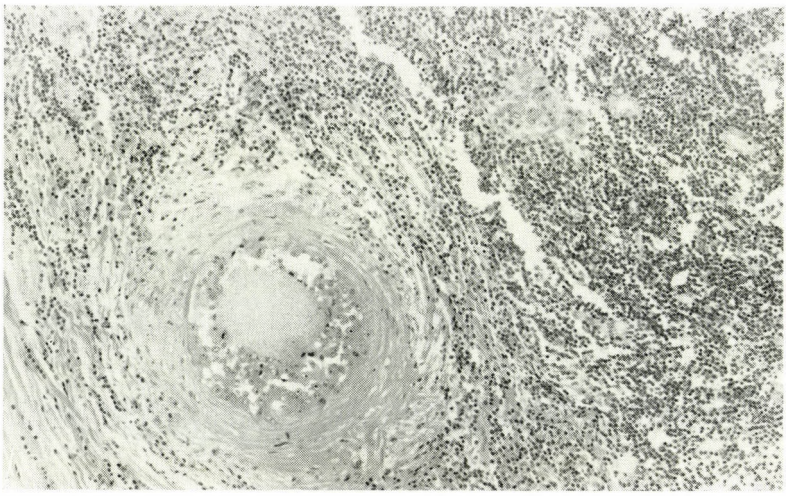

Fig. 7. Metastasis of lymphatic node. Inguinal lymphatic node. Metacrylate-embedded, H. and E. ×224

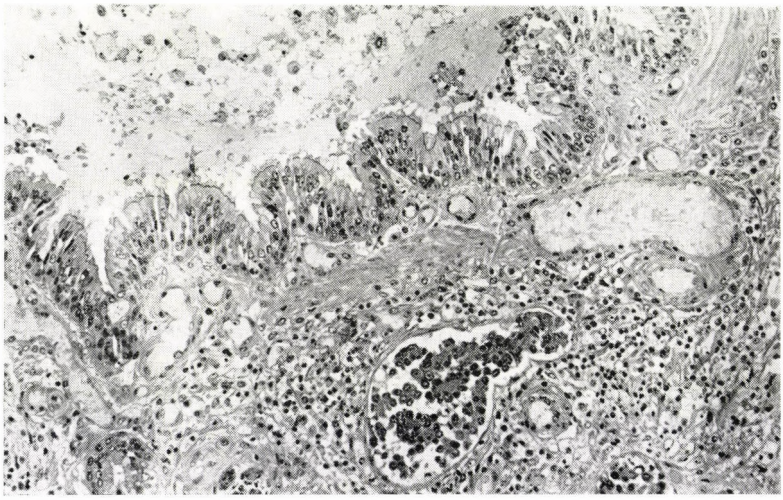

Fig. 8. Metastasis in the lung. Metacrylate-embedded, H. and E. ×308

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EFFECT OF THYROTROPIN (TSH) TREATMENT ON THE VITAMINS A AND E AND LIPID-PEROXIDE STATUS OF DOMESTIC FOWL

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Thyrotropin (TSH) treatment (3000 mU/day/kg body weight intramuscularly) was found to have an effect, through the activation of the thyroid gland, on the lipid (cholesterol) and lipid-soluble vitamin (vitamins A and E) contents of the blood plasma in domestic fowl. The vitamin A and E content of the liver also decreased as an effect of treatment. The lipid-peroxide value, as measured by the malonyldialdehyde (MDA) content of the plasma and the liver, increased as well as the activity of two important free-radical-eliminating enzymes, catalase and glutathione peroxidase.

Keywords. Thyroxine, lipid-peroxide, vitamin E, domestic fowl.

The effect of thyrotropin (TSH) on thyroid activity and thyroid hormone secretion (Kaneko et al., 1969) as well as the interrelationships between thyroid hormones and the lipid metabolism are well-known from the literature. The lipolytic activity of thyrotropin (Butcher et al., 1968) is based on the activation of the cAMP-dependent lipase system. The thyroid hormones negatively correlate with the lipid and cholesterol content of the blood plasma (Fraser, 1954). The fat-soluble vitamins (e.g. vitamins A and E) also correlate with the lipids. Many investigators have suggested a special interaction, an antagonism, between the quantity of vitamin A and thyroid hormones (Wasserman, 1971; Bhat and Cama, 1978) and also between that of vitamin E and thyroid hormones (Wheeler and Perkinson, 1949; Postel, 1956). Thyroid hormones have an effect on the glutathione-dependent free-radical scavenger system as well. Some previous investigations have revealed the effect of thyroid hormones on glutathione-reductase (Menendez, 1974) and superoxide dismutase (Petrović et al., 1982) enzyme activities.

Materials and methods

Twenty-four weeks old broiler hybrid laying hens (Shaver Starcross) were used. A daily dose of 3000 mU/kg body weight of thyrotropin (Ambion inj. Organon, Oss) was given intramuscularly to 10 birds over five days. The daily dose was ten times the daily average production reported in the literature

(Newcomer and Huang, 1974). Control birds (n = 10) were given saline only. The hens received a commercial laying hen diet ad libitum. The room temperature was 23±0.5 °C and the light period 12 h.

Blood was withdrawn daily from the cubital vein into heparinized tubes approximately one hour after the beginning of the light period, for thyroid activity shows diurnal variations (Muray et al., 1981). The injection was performed at the same time. Thyroid activity was determined by measuring the thyroxine level of the plasma. For the determination of thyroxine a specific radioimmunoassay (RIA) kit developed for human diagnostic purpose was used (Institute of Isotopes of the Hungarian Academy of Sciences, Budapest).

Plasma cholesterol was determined by the Liebermann–Burchard reaction (Zöllner and Eberhangen, 1965). Vitamin A derivatives (retinol, retinyl esters) in the plasma were separated by the method of Vahlquist (1974), and their quantity was measured with trichloroacetic acid chromogen (Bárdos, 1975). The vitamin E in the plasma was measured by the method of Bieri (1975). At the end of the experiment the birds were killed by decapitation, and the thyroid weight as well as the vitamin A and E content of the liver were measured. Vitamin A content of the liver was determined by the method indicated above. Vitamin E was measured as described by Haacker (1977).

The lipid peroxide value was determined by measuring the thiobarbituric-acid-reactive products in the plasma and in the liver (Placer et al., 1966; Uchiyama and Mihara, 1978), and expressed in malonyldialdehyde (MDA) content. The standard was 1,1,3,3-tetraethoxypropane (Fluka A. G., Basel). Catalase (E. C. 1.11.1.6) activity was measured by the method of Beers and Sizer (1952) and the activities are given in Bergmeyer units (B. U.). One B. U. is the catalase activity decomposing 1000 mg H_2O_2/min. Glutathione peroxidase (E. C. 1.11.1.9) enzyme activity was measured in a tenfold haemolysate prepared from red blood cells according to Günzler et al. (1974). The unit of glutathione peroxidase activity expresses the number of μmol NADP oxidized per min, as measured at 340 nm. Enzyme activities were related to the protein content, which was determined by the Biuret method.

For the statistical analysis of results Student's t test was used.

Results and discussion

The five-day TSH treatment resulted in an increase of the thyroid weight (Table III).

The thyroxine (T_4) level of the blood plasma increased by the second day of treatment and decreased thereafter, which seems to be a typical waning effect of prolonged, systemic TSH treatment (Table I). The obtained plasma thyroxine values, which are numerically higher than those reported in the

literature, are presumably due to the use of the RIA kit developed for human diagnostic purpose.

The total cholesterol level of the plasma decreased in the treated group from the first to the third day of treatment ($P < 0.05$). It is a well-known effect of thyroid hyperfunction that an enhanced thyroid hormone secretion suppresses the plasma cholesterol level (Table I).

The quantity of vitamin A derivatives (retinol, retinyl esters) in the plasma showed various changes as a result of treatment (Table I). Retinol, which is the biologically active form of vitamin A in most tissues (Goodman, 1980), decreased by the second and third days of treatment. It appears that this effect depends partly on the release of thyroxine, partly on the dose of TSH. It is known that different doses of hormones produce different, sometimes opposite, effects. The effect of thyroid hormones on vitamin A metabolism was investigated earlier (Bárdos and Mézes, 1981). At that time it was found that this was an antagonistic relationship and depended upon hormone dosage.

The quantity of retinyl esters also showed a decrease as an effect of treatment. This effect was based upon the inhibiting effect of thyroid hormones on the carotene–vitamin A conversion in the intestinal wall (Abelin, 1933). As compared to the control, the decrease was significant ($P < 0.01$) on the second and third days of treatment, indicating that also this change was related to thyroxine release.

In the TSH-treated group the vitamin E content of the plasma also decreased significantly by the second day (Table II). It was shown earlier that enhanced thyroid hormone secretion led to lower vitamin E levels in the plasma (Postel, 1956). Wheeler and Perkinson (1948) found that the higher metabolic rate resulted in increased vitamin E requirements, and therefore also the plasma vitamin E level decreased. In the TSH-treated group an appreciable decrease ($P < 0.05$) was found in the vitamin E content of the liver (Table III) as well.

The lipid-peroxidative processes were investigated by measuring the level of MDA, which is one of the end-products of the secondary oxidation of lipids. It was found that in the TSH-treated group the plasma MDA content increased ($P < 0.05$) from the fourth day of treatment (Table II). These changes showed that TSH treatment had a dose-dependent effect on lipid peroxidation, and the increase of the plasma MDA level was not closely related to the actual thyroxine level.

The catalase enzyme activity of the plasma was higher in the treated group ($P < 0.05$) on the second and third days of treatment, as compared to the control (Table II).

The glutathione peroxidase activity of the haemolysate increased from the third day of treatment (Table II). The changes found in the enzyme activities suggest that the organism can eliminate the oxygen free-radicals, first by the activation of catalase enzyme which can decompose hydrogen peroxide,

Table I

Plasma thyroxine, cholesterol and vitamin A levels during TSH treatment

Day of treatment	Group		Thyroxine (nmol/l)	Cholesterol (mmol/l)	Retinol	Retinyl esters
					(μmol/l)	
0	Treated	$\bar{x}$	57.43	3.74	2.50	9.40
		$s\pm$	12.30	0.48	0.98	2.70
	Control	$\bar{x}$	52.43	2.97	2.37	9.70
		$s\pm$	11.00	0.58	0.87	2.90
1	Treated	$\bar{x}$	147.17***	2.31*	2.10*	8.80
		$s\pm$	26.50	0.47	0.40	1.10
	Control	$\bar{x}$	49.43	3.28	2.70	7.10
		$s\pm$	18.79	0.20	0.10	2.20
2	Treated	$\bar{x}$	251.33***	2.28*	1.20**	7.00**
		$s\pm$	52.69	0.31	0.40	2.10
	Control	$\bar{x}$	46.83	3.31	1.90	12.30
		$s\pm$	21.60	0.40	0.10	1.30
3	Treated	$\bar{x}$	107.33**	1.98*	1.60	6.50**
		$s\pm$	23.20	0.48	0.20	0.60
	Control	$\bar{x}$	61.33	3.64	1.50	9.00
		$s\pm$	13.47	0.88	0.70	1.60
4	Treated	$\bar{x}$	89.00	2.98	2.00	2.70
		$s\pm$	23.68	1.02	0.50	1.10
	Control	$\bar{x}$	64.29	2.95	1.90	3.40
		$s\pm$	16.50	1.30	0.10	0.10
5	Treated	$\bar{x}$	71.66	2.94	1.50	5.90
		$s\pm$	27.40	0.88	0.20	1.50
	Control	$\bar{x}$	60.03	3.96	2.70	5.80
		$s\pm$	15.27	1.20	0.70	1.90

* = $P < 0.05$ ** = $P < 0.01$ *** = $P < 0.001$

and subsequently by the activation of glutathione peroxidase which can decompose not only hydrogen peroxide but organic peroxides (mainly lipid-hydroperoxides) as well. The elevation of the plasma MDA level seems to support this hypothesis.

No significant alteration occurred in the MDA content of the liver, which means that the enzymatic adaptation of hepatocytes is sufficient for the elimination of oxygen free-radicals.

The results indicate that TSH treatment has an effect not only on lipid metabolism, but on the metabolism of lipid-soluble vitamins (vitamins A and E) as well. In domestic fowl, this effect extends also to the lipid-peroxide status. The effect is based on the activation of the thyroid gland, which was demonstrated by measuring the T_4 level of the plasma.

Table II

Plasma vitamin E and malonyldialdehyde content, catalase activity and haemolysate glutathione peroxidase activity

Day of treatment	Group		Vitamin E (mmol/l)	TBA-reactive plasma products (MDA nmol/ml)	Catalase (B. U./g prot.)	Glutathione-peroxidase (U/g prot.)
0	Treated	$\bar{x}$	0.61	7.44	17.83	1.69
		$s\pm$	0.10	1.34	7.51	0.26
	Control	$\bar{x}$	0.62	5.98	20.27	1.95
		$s\pm$	0.12	0.88	4.50	0.42
1	Treated	$\bar{x}$	0.63	5.18	19.66	2.38
		$s\pm$	0.03	1.23	3.54	0.92
	Control	$\bar{x}$	0.64	5.21	18.49	2.16
		$s\pm$	0.06	1.14	3.16	0.69
2	Treated	$\bar{x}$	0.52*	5.89	25.10*	3.62
		$s\pm$	0.03	0.73	4.54	0.85
	Control	$\bar{x}$	0.47	6.64	19.55	3.38
		$s\pm$	0.03	1.58	2.22	0.52
3	Treated	$\bar{x}$	0.47	5.92*	20.99*	5.51*
		$s\pm$	0.04	0.46	2.38	0.94
	Control	$\bar{x}$	0.48	7.77	17.10	2.15
		$s\pm$	0.05	1.21	2.29	0.28
4	Treated	$\bar{x}$	0.52	13.63*	19.45	2.99*
		$s\pm$	0.03	2.96	8.12	1.11
	Control	$\bar{x}$	0.49	10.37	15.21	1.93
		$s\pm$	0.04	2.21	6.07	0.56
5	Treated	$\bar{x}$	0.54	12.41*	16.19	2.14*
		$s\pm$	0.06	2.76	2.22	0.50
	Control	$\bar{x}$	0.54	7.73	15.66	1.44
		$s\pm$	0.04	2.37	1.51	0.34

* = $P < 0.05$

Table III

Thyroid weight and vitamin A, vitamin E and malonyldialdehyde content of liver after TSH treatment

	Treated	Control
Thyroid weight (mg)	360.31 ± 51.85**	204.33 ± 62.26
Liver vitamin A (mmol/g)	0.71 ± 0.19*	1.07 ± 0.29
Liver vitamin E (mmol/g)	0.96 ± 0.29*	2.69 ± 1.03
Liver malonyldialdehyde (μmol/g)	0.72 ± 0.24	0.46 ± 0.29

* = $P < 0.05$ ** = $P < 0.01$

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OCCURRENCE AND SOME CHARACTERISTICS OF THE IgG RECEPTORS OF THE SMALL INTESTINAL MUCOSA: IN VITRO EXPERIMENTS

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Binding of ^{131}I-labelled IgG to the membrane fraction of small intestinal mucosal homogenates of young specimens of various species and some factors influencing this binding were studied in vitro. Specific binding was demonstrated by competition with unlabelled IgG.

Bovine IgG_1 and IgG_2 were not bound specifically to the epithelium of the proximal and distal small intestine of the newborn lamb, piglet, cat, rabbit and guinea-pig and of the newly-hatched chicken. Chicken IgG was not bound specifically to the intestinal epithelium of the newly-hatched chicken and of the suckling rat.

The epithelium of the proximal small intestine of the 18-day-old rat bound bovine IgG_2 specifically, but little or no bovine IgG_1 was bound. The specific binding could be blocked by adding unlabelled ovine serum, porcine serum or bovine IgG_2 previously, but not by bovine IgG_1 or by the newborn piglet serum devoid of gamma globulin.

In the proximal small intestine of the rat, the specific binding of bovine IgG_2 declined rapidly between days 17 and 23. It is known that the absorption of antibodies from the milk ceases between days 19 and 21 in this species. In the distal part of the small intestine, the binding of bovine IgG_2 was very low throughout.

The results of the present experiments extend the earlier findings about the characteristics of intestinal IgG receptors mediating the *selective* transmission of IgG in the suckling rat, and indicate the lack of IgG receptors in species with *non-selective* IgG transmission (piglet, lamb, kitten) and in species which are unable to transmit IgG neonatally across the gut (rabbit, guinea-pig, chicken).

Keywords. IgG receptors, small intestine, rat, pig, sheep, cat, rabbit, guinea-pig, chicken, bovine IgG, chicken IgG.

Brambell (1966) was the first to suggest that intestinal immunoglobulin transmission was mediated by specific receptors in the brush border membrane of the enterocytes of the suckling rat. Later the presence of F_c receptors was confirmed, by means of labelled immunoglobulins, in the proximal part of the small intestine. The receptors were found to be specific for IgG while showing different affinities for IgG subclasses and for IgGs of different species (Jones and Waldmann, 1972; Mackenzie, 1972; Rodewald, 1973). Similar receptors were found in the small intestine of the suckling mouse (Guyer et al., 1976).

In the present work the occurrence, disappearance and some characteristics of intestinal IgG receptors were studied in a simple in vitro system.

Materials and methods

Preparation and labelling of IgG

Bovine IgG was prepared by salt precipitation repeated three times and separated to subclasses on a DEAE-cellulose column (Milstein and Feinstein, 1968) in immunochemical purity. Chicken and human IgGs were Cohn-fractions with a 90% IgG content. Bovine and chicken IgG preparations were labelled with carrier-free ^{131}I in the presence of Chloramine T. Labelled IgG was separated from unbound activity on a Sephadex G-25 column after reduction with Na metabisulphite.

Preparation of crude mucous membrane fraction

Proximal and distal small intestines of 7 species (rat, guinea-pig, rabbit, cat, pig, sheep and chicken) were sampled at different ages. In the chicken, jejunal and caecal samples were obtained. Before killed, the newborn animals were not allowed to suckle and the older ones were starved for at least 6 h in order to provide time for the disappearance of IgG from the gut. The animals were killed by bleeding. After slitting, the intestine was gently rinsed with saline and then frozen in 0.1 M, pH 6.0 acetate buffer. The day after, the intestine was stirred vigorously for 5 min with a glass rod at room temperature. The intestinal wall was then discarded and the mucous membranes were centrifuged at 6000 *g* for 15 min. The sediment containing the crude membrane fraction was suspended in acetate buffer and volumes representing 20 mg dry matter were pipetted into plastic centrifuge tubes.

Binding experiments

Five μg labelled IgG ($\sim$12 kBq) was added to about 20 mg (d. m.) crude membrane fraction and kept at room temperature while shaken periodically. After 15 min the mixture was washed with 2 ml acetate buffer 3 times, each time centrifuging the membrane fraction at 6000 *g* for 15 min. The remaining activity (total binding) was expressed in per cent of the original activity. The total binding was the sum of the specific and nonspecific binding. In the control tubes 500 μg unlabelled human IgG preceded the labelled IgG. The control value was considered nonspecific binding, and the specific binding was calculated by subtracting the control value from the total binding. The unlabelled IgG slightly reduced the nonspecific binding, too, but the reduction never surpassed 1%, as determined with adult rat's intestine. In experiment 1 the values were corrected accordingly. Numerical values of experiments 2 and 3 cannot be compared.

In experiment 1, pooled intestines of 12 chickens were studied; the intestines of rabbits, guinea-pigs and cats were pooled in each litter; piglets and lambs were tested individually. At least 4 animals or 4 litters were studied in each species. In experiment 2, each experimental series was done with pooled intestines of 18-day-old rats. In experiment 3, the intestines were pooled by pairs.

Results

Experiment 1

The binding of bovine IgG_1 and IgG_2 to the epithelium of the proximal and distal portions of the small intestine was studied in 7 species in vitro. The species and their ages were as follows: 18-day-old and adult rats, newborn and 10-day-old rabbits, 1½-day-old guinea-pigs, 1½- and 5-day-old kittens, pig fetuses in the last third of pregnancy, newborn piglets and lambs, and newly-hatched chickens.

No specific binding of bovine IgG was detected, except in the 18-day-old rat, which was studied in detail in experiment 2. Chicken IgG did not bind specifically to the intestinal epithelium of the newly-hatched chicken and of the 18-day-old rat.

Experiment 2

Specific binding of bovine IgG_1 and IgG_2 was studied in different circumstances with the epithelium of the proximal small intestine of 18-day-old rats (Table I).

Table I

Specific in vitro binding of bovine IgG to the membrane fraction of the epithelium of suckling rat's proximal small intestine: results of a representative experiment

Membrane + bovine IgG_1*		0.4%
Membrane + human IgG + bovine IgG_1*		0 %
Membrane	+ bovine IgG_2*	6.0%
Membrane + human IgG	+ bovine IgG_2*	0 %**
Membrane + ovine serum	+ bovine IgG_2*	0 %
Membrane + porcine serum	+ bovine IgG_2*	0 %
Membrane + newborn pig serum	+ bovine IgG_2*	5.6%
Membrane + bovine IgG_1	+ bovine IgG_2*	5.0%
Membrane + bovine IgG_2	+ bovine IgG_2*	0 %

Each value represents the mean of two measurements.
The amounts used of the respective reagents per tube: membrane 20 mg (d. m.), labelled bovine IgG 5 μg, unlabelled bovine IgG 100 μg, human IgG 500 μg and serum 25 μl.

* = ^{131}I-labelled IgG
** = reference value

Experiment 3

In the proximal half of the small intestine of the rat pups, binding of bovine IgG_2 declined steeply between days 17 and 23 of life (Fig. 1). In the distal portion no conclusive demonstration of the changes was possible, due to the low initial values.

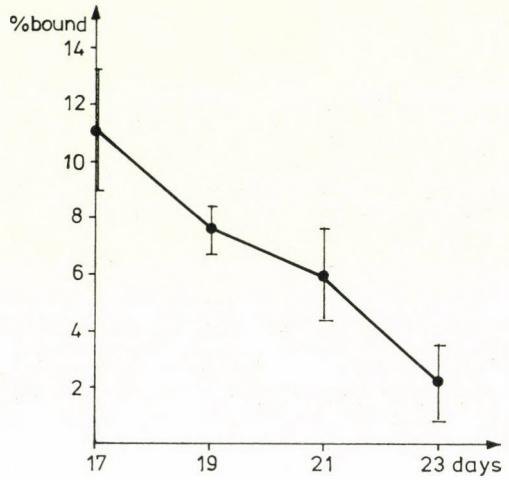

Fig. 1. Binding of bovine IgG_2 to the proximal small intestinal epithelium of the rat between days 17 and 23 of postnatal life. Averages for 4 litters ± standard error

Discussion

All of our IgG-binding tests were negative, with the exception of suckling rats (Experiment 1). The newborn animals born without gamma globulin (piglets and lambs) were not allowed to suckle, thus, the contamination of their intestines with IgG of the blood could be excluded. In the other species contamination with serum IgG was possible, however, the animals were exsanguinated and the intestines were prepared in the same way as those of the rat pups reacting positively.

Until now intestinal receptors binding native IgG could be demonstrated only in species that transported IgG selectively (rat and mouse). The small intestine of piglets, lambs and kittens transports large amounts of IgG on the first 2 days of life, but without any apparent selection between proteins (Brambell, 1958). Accordingly, we failed to detect specific IgG receptors in these species; neither did the nonspecific binding exceed that of the suckling rat. In the rabbit and guinea-pig no substantial quantities of IgG are transmitted by the gut postnatally; the binding experiments were negative accordingly. The negative results with chicken intestines are in tune with the demonstration

that in this species antibodies are transmitted via the yolk sac epithelium (Brierley and Hemmings, 1956) and not through the intestinal mucosa (Clarke and Hardy, 1970).

The IgG of the ruminants, as compared to most of the other IgGs of mammalian origin, is poorly absorbed by the intestine of the suckling rat (Brambell, 1966) and binds poorly to the intestinal receptors (Jones and Waldmann, 1972). Bovine IgG is composed of two subclasses, and the intestine of the suckling rat transmits the more basic fraction preferentially (Jones, 1976). In accordance, we found the bovine IgG_2 of relatively high pK to bind specifically to the proximal intestinal epithelium of the suckling rat, while the bovine IgG_1 of lower pK bound slightly or not at all (Experiment 2). The bovine IgG_1 did not compete substantially with bovine IgG_2 for the receptors (Table I). A contrasting behaviour was observed during the production of bovine colostrum (Brandon et al., 1971): the udder of the cow secretes much more IgG_1 than IgG_2.

In the present experiments (Table I) sheep and pig sera inhibited the specific binding of labelled IgG, but the newborn piglet sera, devoid of gamma globulin, did not.

In the suckling rat, at the end of the third week of life, the intestinal epithelium alters its differentiation in an "all or none" way in the distal half of the small intestine as a consequence of the elevation of corticosterone production (Halliday, 1959; Daniels et al., 1972). The new cells replace the previous, vacuolized ones on the 19th and 20th day (Clarke and Hardy, 1969; Baintner and Veress, 1970). Although no marked morphological changes were seen in the proximal small intestine, the changes of the enzyme pattern (Baintner and Veress, 1970), the cessation of IgG transmission on days 19 and 20 (Halliday, 1956), and the decrease of the binding of IgG between days 17 and 23 (Fig. 1) indicate that the redifferentiation of the epithelium extends to the proximal small intestine, too. However, in the present experiment the changes were slower than the enzymatic and morphological changes investigated in our earlier experiments (Baintner and Veress, 1970).

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STUDIES ON THE AVAILABILITY, TRANSPORT AND UTILIZATION OF OXYGEN IN LOW- AND HIGH-FLOW SEPTIC SHOCK IN CALVES

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Availability, transport and utilization of oxygen were studied in 20 clinically healthy calves exhibiting hypokinetic (low-flow), hyperkinetic (high-flow), or a combination of both, flow states at different stages of experimental septic shock. Arterial P_{O_2} (partial pressure of oxygen in blood) decreased significantly at terminal stages but oxygen saturation of arterial haemoglobin remained almost unaffected and CaO_2–CvO_2 (arterial and venous oxygen contents) had a tendency to decrease in all shock states. The P_{50} values (the P_{O_2} at which blood is 50% saturated with oxygen) had a tendency to decrease only at late shock with low- and high-flow, while the same was true at all stages in combined shock. Average P_{O_2} increased only in combined high- and low-flow septic shock. There was reduction in O_2 delivery at early (9.2%) and late (16%) shock stages in the low-flow group, while in the high-flow group O_2 delivery increased at both stages (17.9% and 40.2%, respectively). In combined high- and low-flow septic shock, O_2 delivery increased at early and decreased at late shock stages. O_2 utilization declined invariably in all forms and at all stages of shock with a great deal of variation in magnitude except at early stages of combined shock, where it increased.

Keywords. Septic shock, oxygen, calf.

Septic shock is still a largely unsolved problem with regard to an understanding of the mechanisms involved and management (Gelin et al., 1980). The weight of this problem is especially enhanced in bovine species, where very few studies on septic shock have been conducted so far. Sporadic evidence obtained from intravenous administration of endotoxins is inadequate since the picture of shock thus produced is markedly different from the clinical septic shock (Perbellini et al., 1978). Singh and Kohli (1980) investigated some basic pathophysiological changes in bovine septic shock. Recently much interest has been attracted by the oxygen transport system during various stress conditions. Apart from the cardiac output and its distribution, blood oxygen content and affinity of haemoglobin for oxygen are the factors upon which oxygen transport to the tissues is dependent. In cattle, responses of the cardiovascular system to septic shock were found to be in three distinct categories (Sahay, 1982). These animals may exhibit hypokinetic dynamics (low-flow state) or hyperkinetic dynamics (high-flow state) or a combination of the two flow states. There does not appear to be any study on the various aspects of oxygen transport system with different flow states in bovine septic shock. An

extrapolation of data on septic shock from one species to another may be misleading since substantial species variations have been recorded (Kuida et al., 1961; Singh and Kohli, 1980). The present investigation was, therefore, undertaken to evaluate the changes in the availability, transport and consumption of oxygen in different flow states of experimental septic shock in calves.

Materials and methods

Twenty clinically healthy, 1 to 1½ years old crossbred calves maintained under isomanagerial schedule were used. Catheterization of carotid artery and jugular vein for collecting blood samples and for haemodynamic measurements, and induction of septic shock by strangulating a segment of jejunum, were accomplished as described earlier from this laboratory (Singh and Kohli, 1980).

The P_{O_2} (partial pressure of oxygen in blood) of arterial (PaO_2) and venous (PvO_2) blood and their pH were measured in a Blood Gas Analyser (Radiometer, Copenhagen, the B. M. E. 33 blood microequipment) at 37 °C. The arterial (SaO_2) and venous (SvO_2) oxygen saturation was derived from Severinghaus nomogram using measured P_{O_2} and pH values. The arterial (CaO_2) and venous (CvO_2) oxygen contents were calculated according to the formula:

$$\text{Oxygen content (ml/100 ml)} = \frac{\text{Hb} \times 1.34 \times S_{O_2}}{100},$$

where Hb = haemoglobin g/100 ml, 1.34 = solubility coefficient of oxygen in haemoglobin, and S_{O_2} = oxygen saturation of blood.

The P_{50} value (the P_{O_2} at which blood is 50% saturated with oxygen) of arterial blood was measured using the factors described by Severinghaus (1966), on a blood gas calculator (type BCG 1, Radiometer, Copenhagen).

Average (Av.) $P_{O_2} = PvO_2 + {}^1/_3 (PaO_2 - PvO_2)$.

Oxygen delivery to the tissues (ml/min) was measured as cardiac output (Q) (L/min) $\times$ CaO_2 (ml/L).

O_2 consumption was measured by using the equation:

O_2 consumption = Q (L/min) $\times$ $CaO_2 - CvO_2$ (ml/L).

Stabilized preshock values of all the parameters were taken to serve as normal control (stage O) of the respective parameters. Since the survival time of individual animals following shock varied widely, the time elapsing between creation of shock and death was divided into five equal stages (stages 1 to 5). Early and late/terminal shock stages corresponded to stages 2 and 5, respectively. The values of O_2 delivery and O_2 consumption for the other stages were extrapolated from the corresponding value of Q. Statistical analysis was done with one-way analysis of variance. For stagewise analysis Student's *t* test was applied.

Results and discussion

Induction of septic shock by strangulating a segment of bowel has been convincingly demonstrated in a similar model (Singh and Kohli, 1980). On the basis of Q and related haemodynamics the ensuing shock in the present study exhibited low-flow state (hypokinetic), high-flow state (hyperkinetic) and combination of low- and high-flow state (combined shock) in 12, 3 and 5 animals, respectively. Accordingly, the data of these groups have been analysed separately to show the effect of flow states on the different parameters of oxygenation during shock stages (Table I). The average survival time after induction of shock in low-flow, high-flow and combined shock was 22, 27 and 29 h, respectively.

It is apparent that arterial P_{O_2} exhibited a significant ($P < 0.05$) fall only in the terminal stage (stage 5) in all the flow states. Contrarily, SaO_2 showed marginal differences and CaO_2–CvO_2 exhibited a tendency to decline in the last stages but its magnitude was greater in combined high- and low-flow shock (Table I). The P_{50} of arterial blood showed statistically significant ($P < 0.05$) decrease at stage 4 in low-flow and from stage 3 onward in high-flow, but it had a tendency to decrease at all stages in the combination group. However, on stagewise analysis the decrease in P_{50} in the latter group was significant ($P < 0.05$) only at stage 3.

Reduction of the oxygen delivery at early (9.2%) and late/terminal (16%) stages of low-flow shock was mainly due to decreased Q. Another possible reason was the observed increased affinity of O_2 for haemoglobin (decreased P_{50} values) at this stage. In such a state oxygen unloading is hampered at the tissue level (Filley, 1971). However, the beneficial effect of the increased affinity of O_2 for haemoglobin was exhibited at the lung level, where in the terminal stages, despite the highly significant fall in P_{O_2} values, oxygen saturation was affected only marginally. The alteration in the affinity of O_2 for haemoglobin was found to be related with the changes in blood pH, CO_2 tension, body temperature and certain organic phosphates (Brenna et al., 1972). It is probable that the interaction of these ligands which affect the oxyhaemoglobin dissociation curve (Wranne et al., 1972) was responsible for the left shift observed in the present study. In combined shock, a significant decrease in P_{50} value was observed in the terminal stages and this left shift should contribute to the low O_2 delivery at this stage. However, a left shift in the high-flow shock was effectively compensated by the appreciably increased Q.

Average P_{O_2} in the present study was calculated to find the relationship between this variable and the CaO_2–CvO_2. This variable is more important than P_{O_2} alone since it is this pressure which drives O_2 from systemic capillaries to the tissues. Consequently, an increased Av. P_{O_2} should drive more oxygen to the tissues and thus increase CaO_2–CvO_2 (Filley, 1971). Interestingly, in the

Table I

Mean ± S. E. of oxygenation parameters in low-flow (A), high-flow (B) and combination of high- and low-flow (C) septic shock in calves

Parameter	Stages of shock					
	0	1	2	3	4	5
Arterial P_{O_2} mm Hg						
A	81.3 ± 3.12	82.1 ± 2.09	80.2 ± 2.12	80.2 ± 2.47	77.8 ± 2.88	69.1 ± 2.17
B	77.5 ± 2.04	77.8 ± 3.48	76.06 ± 4.99	74.8 ± 0.50	75.8 ± 3.30	66.4 ± 4.78
C	93.8 ± 8.34	90.66 ± 3.62	81.46 ± 3.06	87.4 ±21.19	87.3 ± 2.11	75.4 ± 2.87
SaO_2%						
A	96.2 ± 0.41	96.5 ± 0.29	96.6 ± 0.30	96.5 ± 0.34	96.0 ± 0.65	94.6 ± 1.05
B	96.1 ± 0.29	96.2 ± 0.93	96.3 ± 0.55	96.5 ± 0.05	96.6 ± 0.44	95.3 ± 1.02
C	96.3 ± 1.16	97.6 ± 0.27	97.2 ± 0.35	97.8 ± 0.47	97.7 ± 0.23	97.1 ± 0.49
CaO_2–CvO_2 ml/100 ml						
A	3.30 ± 0.27	3.42 ± 0.23	3.50 ± 0.27	3.39 ± 0.20	3.04 ± 0.36	2.87 ± 0.38
B	3.79 ±0.52	3.64 ±0.38	3.94 ±0.61	3.03 ±0.67	3.44 ±0.33	3.00 ±0.08
C	3.85 ±0.33	2.90 ±0.91	3.60 ±1.2	3.57 ±0.48	3.17 ±0.5	2.10 ±0.66
Arterial P_{50} mm Hg						
A	26.23 ± 0.11	26.12 ± 0.08	25.79 ± 0.19	25.91 ± 0.14	25.77 ± 0.12	25.88 ± 0.28
B	26.20 ± 0.10	26.23 ± 0.08	26.0 ± 0.11	25.66 ± 0.08	25.43 ± 0.28	25.66 ± 0.06
C	26.62 ± 0.28	26.4 ± 0.18	25.72 ± 0.42	25.22 ± 0.25	25.65 ± 0.17	25.27 ± 0.42
Average P_{O_2} mm Hg						
A	51.4 ± 1.49	51.0 ± 1.21	50.08 ± 0.89	51.7 ± 1.99	52.9 ± 2.46	52. 0 ± 2.22
B	49.1 ± 1.25	48.5 ± 0.30	47.2 ± 1.57	51.2 ± 0.93	50.2 ± 2.01	48.9 ± 1.61
C	50.1 ± 4.4	64.2 ±10.9	62.1 ±12.7	60.9 ± 8.2	61.4 ± 4.6	68.1 ± 8.4
O_2 delivery ml/min						
A	1274.3 ± 77.9	1215.5 ± 76.6	1156.7 ± 75.4	1113.2 ± 73.8	1092.4 ± 73.0	1070.8 ± 71.4
B	1179.2 ±144.7	1284.7 ±186.1	1390.6 ±233.7	1521.7 ±231.6	1587.3 ±227.6	1652.9 ±229.6
C	1236.1 ±166.2	1334.8 ±177.5	1436.1 ±193.8	1220.9 ±148.3	1171.8 ±202.9	1167.0 ±206.0

Parameter	Stages of shock					
	0	1	2	3	4	5
O_2 consumption ml/min						
A	346.1 ± 27.9	327.1 ± 26.7	274.3 ± 22.9	261.4 ± 23.1	251.6 ± 17.9	244.6 ± 22.2
B	369.3 ± 81.4	362.9 ± 79.6	362.6 ±113.3	357.2 ± 75.4	354.5 ± 64.3	351.9 ± 53.4
C	391.5 ± 33.4	431.6 ± 48.8	468.9 ± 80.3	329.4 ± 50.6	228.7 ± 46.7	228.6 ± 44.4

Abbreviations: P_{O_2} = partial pressure of oxygen in blood; SaO_2 = arterial oxygen saturation; CaO_2 = arterial oxygen content; CvO_2 = venous oxygen content; P_{50} = the P_{O_2} at which blood is 50% saturated with oxygen.

calves of the present study, an inverse relationship was observed. Decreased O_2 consumption, as will be discussed below, and left shift in oxyhaemoglobin dissociation curve should be the two possible reasons for such an abnormal relationship between Av. P_{O_2} and CaO_2–CvO_2. Further, this also suggests a typical bovine species variation in a septic episode.

The fall in O_2 consumption with low-flow state was greater (9.8% and 23.9% at early and late shock stages, respectively) when compared to O_2 delivery. In high-flow shock, despite an increase in the O_2 delivery at early (17.9%) and late stages (40.2%), O_2 consumption fell by 1.8% and 4.7%, respectively. Though this decrease in O_2 utilization is marginal, it certainly indicates that during septic shock the cells do not utilize O_2 even if the latter is transported in greater quantity. In septic shock there is a direct competition for O_2 between bacterial cytochrome and the muscle mitochondria (Stevens, 1979) as a result of which there is a fall in O_2 consumption, which disturbs cellular function. Irreversible shock stage follows when appreciable numbers of mitochondria become nonfunctional (Stevens, 1979). Therefore, it is apparent that availability and transport of O_2 affect O_2 utilization in a very limited form in the septic shock in the bovine. However, no explicit explanation can be provided for increased O_2 utilization in early stages with hyperkinetic phase of combined shock in this study.

A relationship has been observed between flow state and survival time in man, where a hyperkinetic circulation associated with septic shock was found to be more detrimental (Cohn et al., 1968). Contrarily, in the present study higher survival time has been recorded with hyperkinetic shock (27 h) and the combined shock where hyperkinetic phase dominated (29 h), as compared to hypokinetic shock, where the survival time was the shortest (22 h). This amply indicates that in septic shock in the bovine a low-flow state is comparatively more detrimental.

A close relationship has also been observed between O_2 consumption and survival time in animals following shock (Crowell and Guyton, 1961). In the

present study, though O_2 consumption did not increase in either high-flow or low-flow shock, a marginal decline only in high-flow shock resulted in a higher survival time than in low-flow shock where the decrease in O_2 consumption was much greater and survival time lesser. However, a similar correlation was not observed in the combined shock, where the highest survival time was observed, yet, O_2 consumption declined significantly ($P < 0.05$) at late shock stage. However, there had been a marginal increase in O_2 consumption at early shock stages only in these animals. It is possible that beneficial effects rendered by increased O_2 consumption, seen only at early stages of combined high- and low-flow shock, might be responsible for sustaining life for a longer period of time despite a greater fall in O_2 consumption at late shock stages.

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EFFECT OF PERIPARTURIENT LIPID MOBILIZATION ON SERUM TOTAL CAROTENE AND VITAMIN A CONCENTRATIONS IN CATTLE

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Carotene and vitamin A levels of the blood plasma were investigated in Black-and-White Holstein-Friesian cows (n = 33), in relation to the degree of disorders in periparturient lipid metabolism. Investigations were started 2 weeks before, and continued up to 5 weeks after, parturition. Milk production of the cows in the previous lactation period was 5700 l on the average. Total carotene, vitamin A, aspartate aminotransferase (AST), albumin, total cholesterol, non-esterified fatty acid (NEFA) and triglyceride values of the blood plasma were determined. Based upon the NEFA concentrations of the plasma, the cows were assigned to three groups: Group I contained cows in the blood plasma of which increasing NEFA levels were measured already in the preparturient period; Group II comprised cows in which increasing NEFA levels were observed only in the postparturient period; in the blood plasma of cows of Group III the NEFA level did not exceed the physiological value.

In the parturient period the physiological decrease of the plasma carotene and vitamin A was enhanced in cows with higher plasma NEFA concentrations. In these cows, involution disorders and dysfunctions of ovarian origin were rather frequent. Presumably, in the periparturient period the pathological lipid metabolism (mobilization) disturbs the transport of carotene and vitamin A, resulting in their greater-than-physiological decrease and prolonged low level in the postparturient period. This may play a role in the delay of the onset of normal cycling in cows affected with disorders of the lipid metabolism (pathological lipid mobilization).

Keywords. Lipid mobilization disorder, fatty liver disease, "fat cow" syndrome, dairy cow, total carotene, vitamin A.

The transition from pregnancy to lactation represents an increased metabolic load for the dairy cow; the degree of the load increases parallel with age and the magnitude of lactation milk production.

The consequent metabolic change may upset the energy balance of the cow. The mobilization of lipids from fat depots may increase to a pathological extent, resulting in an elevation of the non-esterified fatty acid (NEFA) level in the blood plasma (Reid et al., 1979). Simultaneously with this, a so-called "fatty liver" may develop because of hepatocellular accumulation of triglycerides (Kapp et al., 1979; Collins and Reid, 1980; Reid et al., 1979).

In cows that developed clinically apparent fatty liver disease or a subclinical disease with laboratory findings indicating increased lipid mobilization post partum, we measured significantly higher NEFA and significantly lower triglyceride plasma levels already 20–30 days before calving than in cows that remained healthy after parturition (Haraszti et al., 1982). Since the liver has an important role in the metabolic processes of cattle, its damage has adverse

consequences as regards the reproductive processes, resulting in a delayed reactivation of the ovary and in a long service period (Haraszti et al., 1984).

It seems to be justified to assume that the periparturient disorders of lipid metabolism may influence the metabolism of the lipid-soluble vitamins, among them carotene and vitamin A. In the present work data collected on this topic are reported.

Materials and methods

Thirty-three clinically healthy Black-and-White Holstein-Friesian cows, previously calved three or more times and being 3–4 weeks before term, were used. The average production of their previous 300-day lactation was 5700 l. Blood samples were taken from the animals: two before calving and two within 15 days after calving, one between postpartum days 16–29 and one 30–45 days post partum. To follow the occurring metabolic changes, the blood plasma parameters described in an earlier paper (Haraszti et al., 1982), viz. aspartate aminotransferase (AST), albumin, total cholesterol, glucose, non-esterified fatty acids (NEFA), triglyceride, ketone, NEFA: triglyceride ratio, albumin: total protein ratio, were determined. The serum vitamin A level was determined by the fluorometric method of Hansen and Warwick (1978) as modified by Blaskovits et al. (1980). Total carotene concentration was measured according to Brubacher and Vuilleumier (1974) after extraction with petroleum ether. The cows were assigned to three groups based upon the NEFA values measured from day 15 before parturition to postpartum day 10, in agreement with our previous experiences. The cows exhibiting increased lipid mobilization already before parturition constituted Group I, those showing an increased rise of the NEFA level only after calving were assigned to Group II (increased postpartum lipid mobilization), the cows that exhibited only a moderate rise of the NEFA level even after calving constituted Group III (Table I). To reveal the correlations, biometrical calculations were made (mean, standard deviation, *t* test, linear regression). By clinical examinations the reproduction parameters were followed in each group.

Table I

Changes in non-esterified fatty acid (NEFA) concentrations, serving as a basis for grouping of the animals

	Days before parturition		Days after parturition	
	15–6	5–1	0–5	6–10
NEFA concentration, mmol/l				
I Lipid mobilization before parturition	> 0.30	> 0.50	> 0.60	
II Lipid mobilization after parturition	< 0.30	< 0.50	> 0.60	
III Normal	< 0.30	< 0.50	< 0.60	

Results

Laboratory findings indicated increased lipid mobilization already before calving in 13 cows (Group I), and only after parturition in 10 cows (Group II). The rise of the NEFA concentration did not exceed the physiological level in 10 cows (Group III). The mean age for the three groups was as follows: Group I: 6.1±1.0 years; Group II: 6.7±0.8 years; Group III: 4.0±0.9 years.

Changes observed in the total carotene and vitamin A concentrations of the serum are shown in Figs 1 and 2. The ratios of the serum total carotene and vitamin A concentration to AST activity, to the albumin: total protein

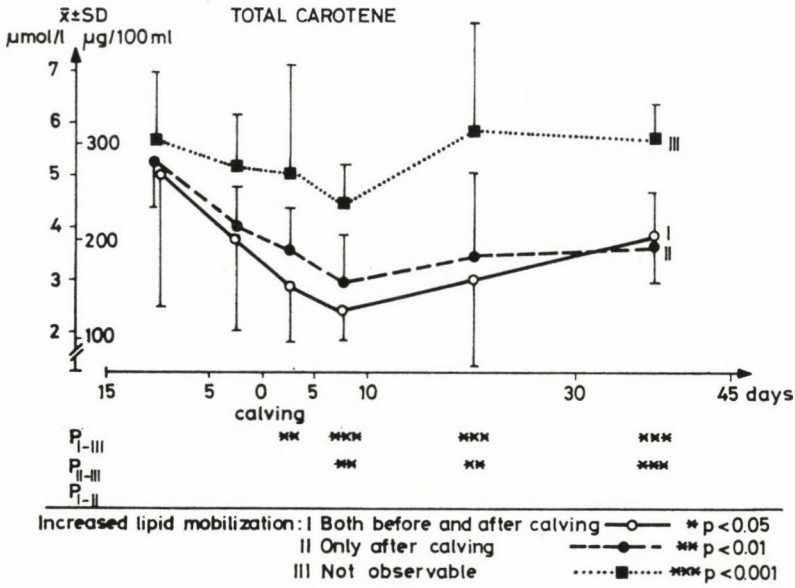

Fig. 1. Serum total carotene levels in the different groups in the periparturient period

ratio, to cholesterol, to NEFA, and to triglyceride concentration are also shown in Tables II—VI. Only values exceeding $r > 0.350$ are given. Correlations reaching at least the significance level of $P < 0.05$ are indicated separately. No or hardly any correlation was found between the blood glucose and ketone level and the vitamin components; therefore, no detailed data are given on this subject.

Involution disorders were of highest incidence in cows in which increased lipid mobilization was observed already before parturition; reproduction-biological parameters were also the least favourable in that group (Tables VII and VIII). The incidence of retention of placenta was 38.5, 10.0 and 10.0% in Groups

Table II

Correlations of the serum total carotene and vitamin A levels with AST activity

	Before parturition			After parturition		
	days					
	15–6	5–1	0–5	6–10	11–30	31–45
AST (GOT)–total carotene						
I			−0.568	−0.966*	−0.310	
II				−0.377	−0.419	
III						
I–II–III						
AST (GOT)–vitamin A						
I						−0.512
II						−0.468
III				−0.458		
I–II–III						−0.400*

Remarks: only $r > 0.350$ values are given, computed by linear regression; * $P < 0.05$

I, II and III, respectively. The proportion of cows conceived before the 150th day was 0 (n = 0), 30 (n = 3) and 50% (n = 5) in Groups I, II and III, respectively.

Table III

Correlations of the serum total carotene and vitamin A levels with the albumin: total protein ratio

	Before parturition			After parturition		
	days					
	15–6	5–1	0–5	6–10	11–30	31–45
Albumin: total protein ratio–total carotene						
I						
II						0.491
III						
I–II–III					0.446*	0.466*
Albumin: total protein ratio–vitamin A						
I						
II						
III						
I–II–III					0.465*	0.379*

Remarks: see Table II

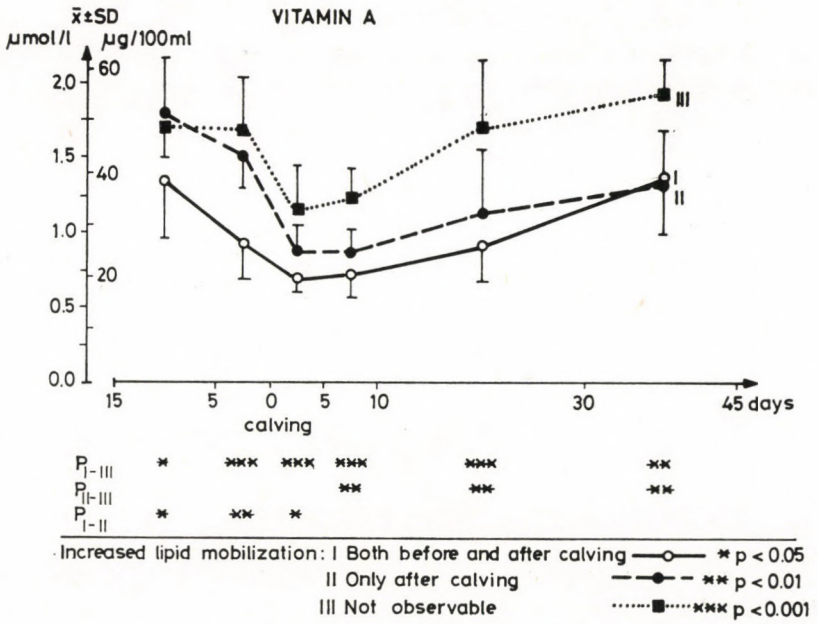

Fig. 2. Serum vitamin A concentrations in the different groups in the periparturient period

Table IV

Correlations of the serum total carotene and vitamin A levels with total cholesterol

	Before parturition			After parturition		
	days					
	15–6	5–1	0–5	6–10	11–30	31–45
Cholesterol–total carotene						
I	0.363	0.877*	0.563*	0.731*	0.546*	0.486*
II		0.698*	0.481	0.789*	0.432	0.361
III		0.378	0.421	0.388	0.371	0.377
I–II–III		0.412*	0.346*	0.691*	0.492	
Cholesterol–vitamin A						
I					0.379*	
II				0.389	0.355	
III		0.321	0.993*	0.420	0.361	
I–II–III		0.401*	0.442*	0.370*	0.391*	0.361*

Remarks: see Table II

Table V

Correlations of the serum total carotene and vitamin A levels with NEFA concentration

	Before parturition			After parturition		
	days					
	15–6	5–1	0–5	6–10	11–30	31–45
NEFA–total carotene						
I	−0.401	−0.371	−0.447	−0.416	−0.588*	−0.489*
II		−0.936*	−0.402	−0.362	−0.377	−0.357
III			−0.357			
I–II–III		−0.459*	−0.388*	−0.503*		
NEFA–vitamin A						
I		−0.381	−0.941	−0.421	−0.381	−0.433
II			−0.492	−0.501	−0.358	−0.384
III						
I–II–III			−0.430*	−0.360*	−0.361	−0.415*

Remarks: see Table II

Table VI

Correlations of the serum total carotene and vitamin A levels with triglyceride concentration

	Before parturition			After parturition		
	days					
	15–6	5–1	0–5	6–10	11–30	31–45
Triglyceride–total carotene						
I	0.367	0.858*	0.459	0.633*	0.356	
II		0.361	0.718*	0.401	0.433	
III			0.436	0.378	0.381	
I–II–III			0.498*	0.447*	0.455*	
Triglyceride–vitamin A						
I					0.432*	0.458*
II		0.359				
III		0.532*				
I–II–III		0.388*	0.368*	0.357	0.386*	0.387*

Remarks: see Table II

Table VII

Incidence of involution complications and ovarian disorders by groups

	Group I n = 13 = 100%		Group II n = 10 = 100%		Group III n = 10 = 100%	
	n	%	n	%	n	%
Involution						
Retention of placenta	5	38.5	1	10.0	1	10.0
Early involution disorders	7	53.8	5	50.0	3	30.0
Late involution disorders[1]	6	46.1	4	40.0	1	10.0
Ovarian function[1]						
Anovulatory cysts	1	7.7	2	20.0	1	10.0
Loss of function or hypofunction	4	30.8	2	20.0	1	10.0

Remark: [1]Between days 30 and 45 post partum, as determined by rectal palpation

Table VIII

Reproduction-biological indices, by groups

	Group I n = 13 = 100%		Group II n = 10 = 100%		Group III n = 10 = 100%	
	n	%	n	%	n	%
Culled (infertility, mastitis)	4	30.8	1	10.0	1	10.0
Inseminated within 150 days	7	53.8	8	80.0	9	90.0
Conceived within 150 days	—	—	3	30.0	5	50.0
Of this, conceived after one insemination	—	—	2	20.0	4	40.0
			$\bar{x} \pm SD$			
Day of first inseminations[1]	72.9 ± 36.3		58.1 ± 17.0		54.8 ± 19.8	
Day of conception[2]	—		47.5 ± 10.5		45.2 ± 7.8	
Number of inseminations performed per one cow[1]	2.0 ± 0.76		2.2 ± 1.17		1.7 ± 0.97	

Remark: [1] The mean of cows inseminated within 150 days
[2] The mean of cows conceived within 150 days

Discussion

Beta-carotene and vitamin A have an outstanding influence on the sexual functions of cattle. It has been reported that in the bovine corpus luteum (CL) only negligible, if any, vitamin A was present, as opposed to the high carotene concentration in the CL (Schultz et al., 1973; Ahlswede and Lotthammer, 1978). The absence of vitamin A from the bovine CL suggests that the effect of vitamin

A on the ovarian functions is not direct; vitamin A rather exerts a favourable effect on the epithelial cells of the endometrium (Brüggemann and Niesar, 1957; Schultz et al., 1973; Anwandter, 1974; Saelzer et al., 1983). Lotthammer et al. (1976) experimentally proved an advantageous influence of carotene on the overtness of oestrous signs and on the duration of oestrus. Carotene deficiency has an especially adverse influence on ovarian function, manifesting itself in a delayed onset of ovulation and a delayed development and hypofunction of the CL. Of course, the anatomically smaller CL produces less progesterone, resulting in conception and nidation disturbances in the first place (Meyer et al., 1975; Schams et al., 1977; Lotthammer et al., 1976). In the bovine CL, the activity of the enzyme converting β-carotene into retinol is double of that found in the intestinal mucosa; it depends on the biological status of the CL, and, correspondingly, it decreases during the regression phase of the CL (Gawienowski et al., 1974; Sklan, 1983). The serum β-carotene level of cows having ovarian cysts is significantly lower than that of cows free from ovarian disorders (Seitaridis, 1963). On the other hand, as opposed to the cited authors, Folman et al. (1979) failed to detect any difference between cows fed a diet poor in β-carotene and control animals in the duration and intensity of oestrus. Vitamin A influences the synthesis of steroid hormones in the adrenal cortex and the thyroxine production of the thyroid (Scheunert and Trautmann, 1976). This observation seems to be supported by endocrinological, metabolic (cortisol, thyroxine, glucose, AST) and histological data, which confirm that in bulls the activity of the thyroid and adrenal cortex decreases as a consequence of carotene deficiency (Lange, 1977; Krudopp, 1979).

According to Schams et al. (1977), carotene influences the functioning of the ovary and progesterone production through its effect on the adrenal cortex and the hypophysis. Carotene deficiency presumably results in an inhibition of the production of certain proteolytic enzymes important in the onset of ovulation; it has a major role in the retention of placenta, development of puerperal disorders, and prolongation of involution (Lotthammer et al., 1976).

In pregnant cows, a change of carotene level in the immediate peripartu-rient period is characteristic. Serum carotene level, being the highest in the 7th–8th month of pregnancy, shows a gradual decrease 2–4 weeks before parturition. This is followed by its abrupt decrease immediately before parturition, reaching the lowest level in the first days following calving (Lotthammer, 1978; Wälchli-Suter, 1978). The degree of decrease may reach 20–30%. From postpartum days 5–7 carotene level rises again. Similar observations were made on the vitamin A and E levels of the serum (Schmid, 1975; Wälchli-Suter, 1978; Dvorák et al., 1977; Hidiroglou and Hartin, 1982). Taking into account that the colostrum is several times richer in carotene and vitamins A and E than the normal cow milk is, the decrease of these substances can be attributed to the colostral period.

Studies on carotene deficiency provide more and more convincing evidence that β-carotene plays a unique, essential role, not replaceable with vitamin A, in the reproductive processes of cattle, and that it has an influence on certain endocrine and metabolic processes (thyroxine, AST, total cholesterol level; Lotthammer, 1978).

For the sake of completeness it should be mentioned that, on the average, 82% of the total carotene content of the serum is β-carotene (Somorjai and Pethes, 1984).

In the present experiments, decrease of total carotene and vitamin A concentration was observed in all the three experimental groups; the decrease started 5–10 days before parturition and the lowest level was reached on postpartum days 3–5 (vitamin A) and 5–8 (carotene). This was followed by a gradual increase. In Group III, where only moderate lipid mobilization occurred after parturition, the degree of the decrease of carotene level hardly exceeded 20%, and by postpartum days 30–45 carotene concentration practically reached the initial level again. In cows showing lipid mobilization already before (Group I) or only after calving (Group II), a marked elevation of the NEFA level and a decrease of the triglyceride and total cholesterol level were found, indicative of lipid mobilization. The simultaneous decrease of the carotene level exceeded 50%. In these cows carotene level was 25 to 30% lower than the initial value even between postpartum days 30 and 45. As compared to values found for the healthy cows of Group III, the difference was very highly significant ($P < 0.001$) in this respect. In Group I the vitamin A level was significantly lower already before calving, and remained low throughout, as compared to that found for Group III. The difference found up to postpartum day 45 in the vitamin A level in cows of Group II was also significant ($P < 0.01$), as compared to the control values.

The vitamin A value found in cows of Group III, which can be considered physiological, reached its lowest level on postpartum days 3–5; this is in accordance with data reported in the literature (Kuhlman and Gallup, 1944; Hidiroglou and Hartin, 1982). The degree of decrease was approximately 33%, while between days 33 and 45 the serum vitamin A concentration was significantly ($P < 0.01$) higher than in Groups I and II.

In the blood, carotene is transported by lipoproteins. In cattle fed diets of varying fat content it was shown that of the three lipoprotein fractions the high-density lipoprotein (HDL) is the store and the most important transporter of β-carotene; about 90% of the β-carotene is bound to this fraction (Patton and Kelly, 1980; Ashes et al., 1982; Kurz et al., 1984; Ashes et al., 1984).

Vitamin A is stored in the liver and transported to the recipient organs as bound to a specific transportation protein (retinol-binding protein, RBP).

Literary data, together with our own investigations, allow us to assume that in the periparturient period the increased lipid mobilization disturbs the

transportation of carotene and vitamin A, resulting in their greater-than-physiological decrease and prolonged low level in the postparturient period. This may play a role in the delay of the onset of normal cycling in cows affected with disorders of the lipid metabolism.

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EFFECT OF FEEDING RAW SOYBEAN FLOUR ON THE DIGESTION OF THE ADULT SHEEP

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The authors fed raw or heated soybean flour to adult Merino sheep and took samples through ruminal and intestinal fistula for determination of inhibitors and enzyme activities.

Ruminal proteolytic activity is not suppressed by soybean inhibitor (Baintner, 1981); moreover, this inhibitor is gradually degraded by rumen microorganisms in vitro. Bowman–Birk inhibitor is of relatively high resistance, while Kunitz inhibitor is rapidly inactivated.

It seems that the forestomachs of ruminants degrade the soybean inhibitor on the one hand, and delay its passage to the intestines on the other. This explains why we failed to noticeably suppress intestinal proteolysis by feeding 200 g raw soybean flour in a single feed. Furthermore, the unimpaired intestinal proteolysis may rapidly inactivate the fraction of soybean lectin and urease that escaped ruminal digestion. These findings explain the lack of significant antinutritive effect of raw soybean in adult ruminants.

By feeding extremely high amounts of raw soybean flour (500 g for a single feed), transitional suppression of intestinal trypsin activity was achieved and free trypsin inhibitor appeared in the jejunum. Measurement of amylase activity served as a control of pancreatic secretion.

In an additional experiment, no binding of fluorescein-labelled soybean lectin to rumen microorganisms could be demonstrated.

Keywords. Soybean flour, intestinal proteolysis, rumen, protease inhibitor, sheep, lectin.

In birds and in monogastric mammals protease inhibitors of soybean inhibit the activity of trypsin and α-chymotrypsin and depress intestinal proteolysis for several hours after a single feed of raw soybean (Lyman and Lepkovsky, 1957; Alumot and Nitsan, 1961; Lepkovsky et al., 1971; Nitsan and Alumot, 1963). Besides protease inhibitors, other antinutritive factors occur in the soybean (reviewed by Liener, 1981). The inclusion of large amounts of raw soybean flour into the ration of monogastric animals or birds retards growth and induces adaptive responses in the function of exocrine pancreas (Ma'ayani and Kulka, 1968; Gertler and Nitsan, 1970; Nitsan and Liener, 1976; Melmed et al., 1976; Crass and Morgan, 1982).

When adult *ruminants*, mostly dairy cows, were fed on full-fat or extracted raw soybean meal, little or no antinutritive effect was observed (Loosli et al., 1961; Perry and MacLeod, 1968; Larson and Schultz, 1970; Daniels et al., 1973). In the present work the causes of these inter-species differences were studied with in vitro and in vivo techniques.

Materials and methods

Animals

Intestinal and rumen fluid samples were taken every hour from two fistulated Merino sheep. The first one was a 2-year-old wether with a fistula in the middle of the small intestine, while the other was a ewe whose fistula was located in the proximal jejunum. Both of them had rumen fistula, too.

Feeding

The sheep were fed grass hay twice daily, supplemented with 400 g concentrate in the morning. Control (a) and experimental (b) periods alternated in the same animals.

a) Full-fat soybean flour was *steam-heated* at 108 °C (1100 mbar) for half an hour and dried in air. This treatment decreased the trypsin inhibitor content of the flour from 30 to 0.5 mg/g (dry matter). This flour mixed with an equal amount of corn flour was fed as control feed.

b) Full-fat *raw* soybean was ground to flour with an equal amount of corn to facilitate grinding. Four hundred gram of the mixed flour was fed together with hay at the start of the experiment and hay alone in the evening. The small particle size of the flour provided large surface for the extraction of inhibitor by biological fluids.

In one of the experiments we tried to feed to a sheep as much raw soybean flour as possible. In the preceding evening the sheep had been starved and in the next morning full-fat, raw soybean flour was given. As soon as the sheep stopped eating, handfuls of grass were repeatedly mixed to the soya. Thereby the 44-kg animal took up 500 g soya within 3.5 h.

In vitro decomposition of soybean inhibitor

Rumen content was taken from fistulated sheep and pressed through a layer of cotton to obtain rumen liquor. A commercial trypsin inhibitor preparation (Reanal, Budapest) containing both Kunitz and Bowman–Birk inhibitors was added to make a *nominal* concentration of 1 mg/ml. The actual initial concentration was approximately the half of this (see Fig. 5). The rumen liquor was pipetted into test tubes, paraffin oil was layered on the top of the aqueous phase to secure anaerobiosis and it was incubated in a 40 °C water bath. A series of tubes was supplemented with glucose in 1 mg/ml concentration, while the rest of the tubes lacked exogenous energy source. Periodically samples were taken, precipitated with an equal volume of 1% trichloroacetic acid, centrifuged, and the inhibitors were determined in the supernate.

Lectin experiments

Soybean lectin was isolated from an aqueous soybean flour extract by affinity chromatography on a Sepharose-bound N-acetyl-galactosamine column. Adsorbed lectin was eluted with a 0.05 M N-acetyl-galactosamine solution and the sugar was removed by gel chromatography on a Sephadex G-25 column. Purified lectin was labelled with fluorescein isothiocyanate.

Rumen liquor was centrifuged at 15,000 *g* for 20 min and the supernate was discarded. The sediment, containing mostly rumen bacteria with a few protozoa and some food particles, was suspended in saline and centrifuged again to remove dissolved carbohydrates. The resuspended sediment was incubated with fluorescein-labelled lectin at room temperature for 15 min, then the unbound lectin was removed by repeated centrifugation of the microorganisms in saline. Washed rumen microorganisms were examined under fluorescence microscope.

Chemical determinations

Samples were kept frozen until use. After thawing, enzymes and inhibitors were assayed in 10,000 *g* supernate.

Trypsin was determined with benzoyl-arginine ethyl ester (BAEE) as substrate (Schwert and Takenaka, 1955). *Chymotrypsin* was measured by the same principle, with benzoyl-tyrosine ethyl ester (BTEE) as substrate. Traces of activity that split trypsin substrate (BAEE) and were resistant to soybean trypsin inhibitor, occurred both in rumen and intestinal fluids, but did not interfere with the determinations significantly.

Trypsin inhibitor and *chymotrypsin inhibitor* were determined in rumen and intestinal fluids by measuring the decrease of activity of a known amount of added trypsin and chymotrypsin, respectively, and expressed as (chymo)trypsin μg inhibited.

The method of Somogyi (1938) was used for the determination of intestinal *amylase*.

Results

Six experiments with 200 g raw soybean (Fig. 1), one experiment with 500 g raw soybean (Fig. 2), two control experiments with 200 g heat-treated soybean (Fig. 3) and four control experiments without soybean feeding were performed. Trypsin was determined in all intestinal samples, amylase, chymotrypsin and trypsin inhibitor in selected periods only.

Trypsin activities highly varied from hour to hour in the jejunum, sometimes reaching differences of several order of magnitude. Chymotrypsin and

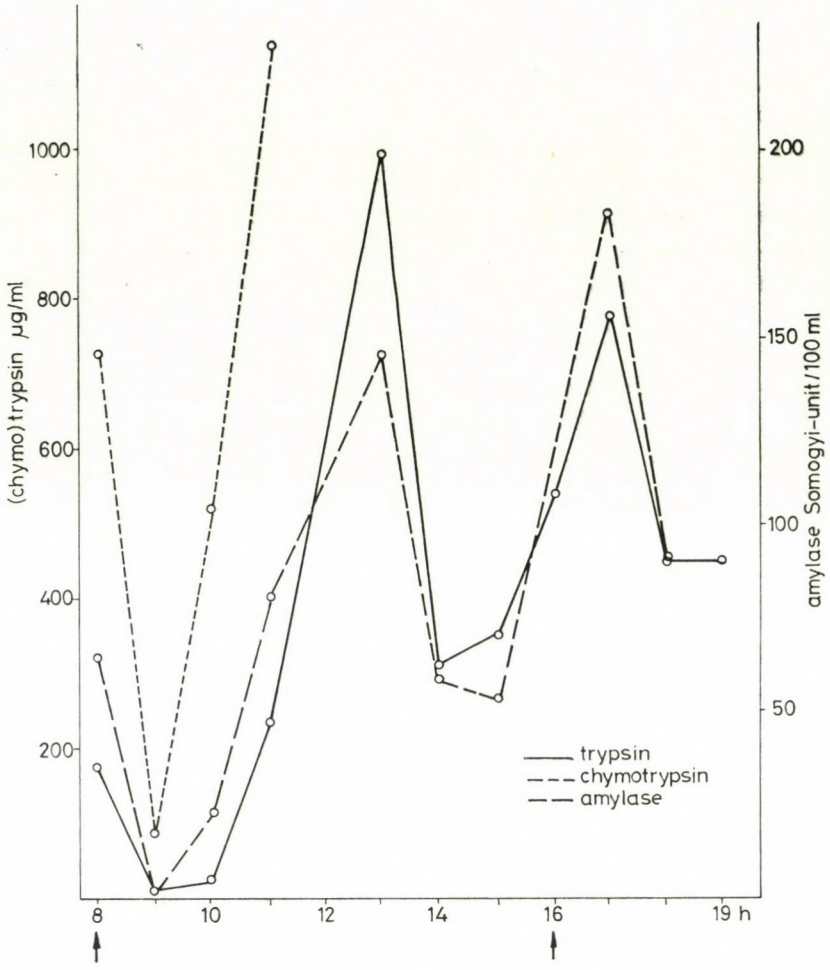

Fig. 1. Activity of digestive enzymes in ovine proximal jejunum after a single feed of 200 g raw soybean flour. No soybean was fed in the afternoon. The start of feeding is indicated by arrows. Free trypsin inhibitor could not be detected. Note the largely parallel alterations of activities. The values are results of single determinations, as in the following figures

amylase activities varied largely parallel with those of trypsin (Fig. 1), except when 500 g raw soybean was fed (Fig. 2). Variation of activities was much less in the middle of the small intestine (Fig. 3) than in the proximal jejunum.

Free trypsin inhibitor could be detected in the rumen liquor after the feeding of raw soybean (Fig. 4); in the intestine only after feeding the highest raw soybean quantity (Fig. 2).

In the in vitro experiments trypsin inhibitor underwent an initial rapid inactivation in the rumen liquor, while the rest of the inhibitor resisted prolonged incubation (Fig. 5). The concentration of chymotrypsin inhibitor showed

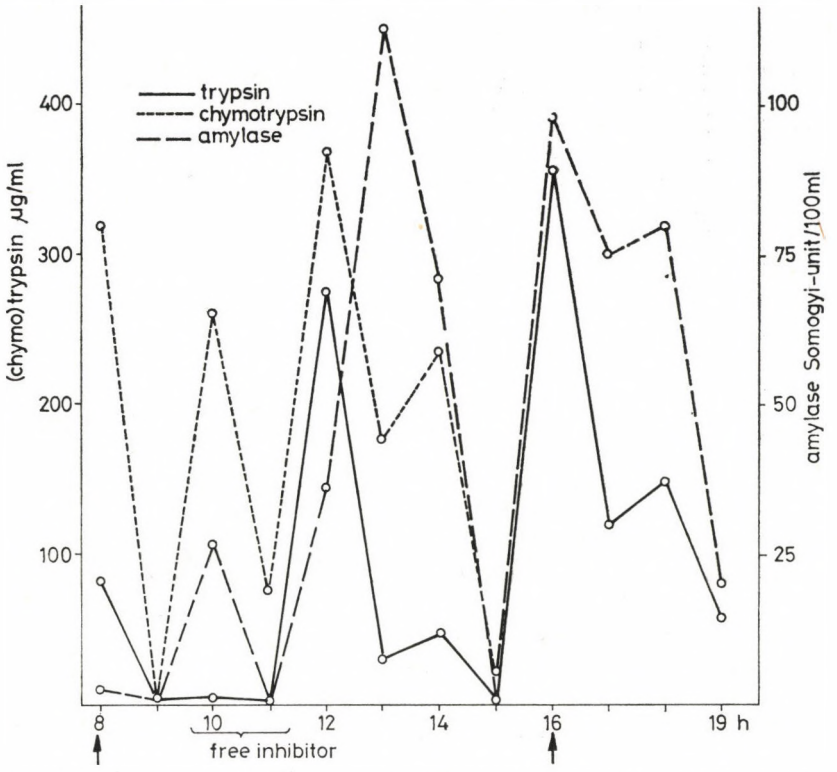

Fig. 2. Similar experiment as in Fig. 1, but with 500 g raw soybean flour. Free trypsin inhibitor was detected in two samples. Note the absence of parallelism of activities, e. g., around the 10 o'clock sample

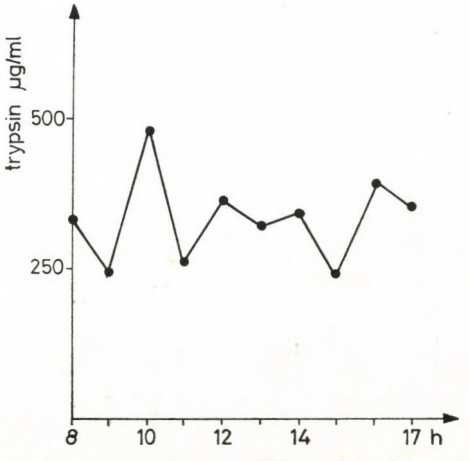

Fig. 3. Trypsin activities in the middle of the small intestine after a single feed of 200 g heated soybean flour (control). Variations are much less than in the jejunum

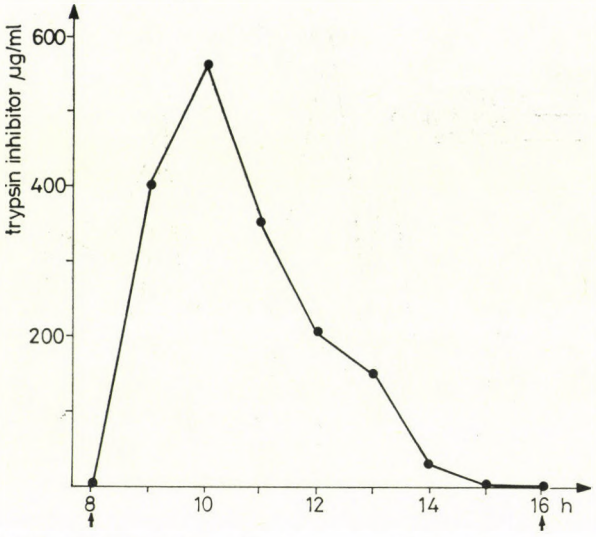

Fig. 4. Trypsin inhibitor concentrations of ovine rumen liquor after a single feed of 200 g raw soybean flour. No soybean was fed in the afternoon. The start of feeding is indicated by arrows

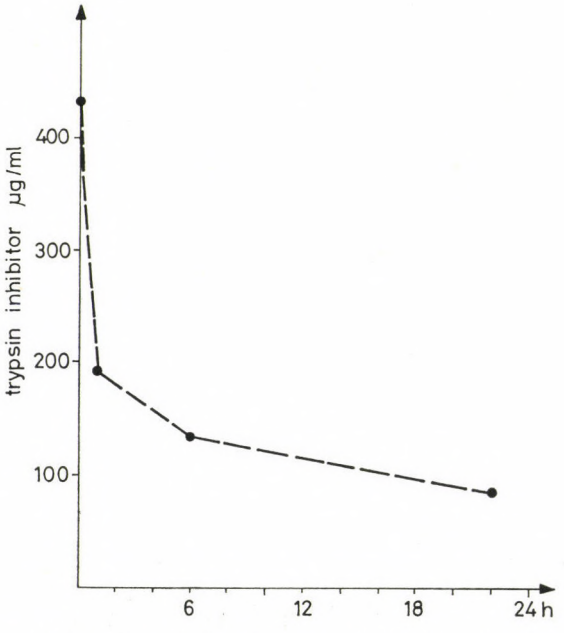

Fig. 5. In vitro decomposition of purified soybean trypsin inhibitor incubated at 40 °C in ovine rumen liquor in anaerobic conditions

little decrease during incubation. Addition of exogenous energy source (glucose) did not affect the rate of inactivation of the inhibitors.

Rumen protozoa exhibited a faint autofluorescence, but fluorescein-labelled soybean lectin was not bound to any of the rumen microorganisms.

Discussion

When 200 g raw soybean flour was fed, one hour after the start of feeding, trypsin activity sometimes fell dramatically to very low values, but several independent observations indicated that this decline was not due to ingested trypsin inhibitor: 1) Trypsin activity and flow rate of intestinal fluid was highly variable throughout. 2) Increased flow rate of intestinal digesta was usually observed after the start of morning feeding, which might have diluted the pancreatic secretions. However, exact measurements of intestinal flow rate were not attempted. 3) The above-mentioned decline in trypsin activity did not occur consistently after feeding raw soybean and it also occurred in some of the control trials. 4) Amylase and chymotrypsin activities varied largely parallel with those of trypsin, although amylase is not inhibited by soybean inhibitors and chymotrypsin is less sensitive to the inhibition than trypsin.

So with moderate amounts of raw soybean flour we could not demonstrate an in vivo effect of the inhibitors on the proteases of the adult sheep, although in vitro ovine trypsin and alpha-chymotrypsin are readily inhibited by aqueous soybean extracts. However, the extreme large (500 g) amount of raw soybean transitionally depressed the intestinal proteolysis, as it does in monogastric animals. In this period amylase and free trypsin inhibitor, but no trypsin could be demonstrated in the intestine (Fig. 2). Traces of BAEE-splitting activity resistant to soybean inhibitor gave the impression of a residual trypsin activity in this period (Fig. 2).

The present in vivo findings explain the lack of antinutritive effect of raw soybean in ruminants. It seems that primarily the presence or absence of forestomachs is responsible for the differential effect of raw soybean on ruminants versus monogastric animals. Ruminal proteolytic activity is not suppressed by soybean inhibitor (Baintner, 1981). Rumen contents dilute the inhibitor and delay its passage towards the intestines; meanwhile it is partially degraded by rumen microorganisms. The rapid disappearance of the inhibitor from the rumen (Fig. 4) indicates that it is rapidly degraded in vivo. So the inhibitor fraction that escaped degradation in the rumen, could not noticeably influence intestinal proteolysis except with extremely high amounts of raw soybean. Young ruminants with not fully developed rumen function, e. g. the growing lambs in the experiments of Dysli et al. (1967), may be more sensitive toward raw soybean.

We may speculate whether the unimpaired intestinal proteolysis may rapidly inactivate the fraction of soybean lectin and urease that escaped ruminal digestion. We suppose that the antinutritive proteins of soybean interact: the suppression of proteolysis is needed for lectin to reach the intestinal mucosa, and the mucosal damage made by lectin is required for urease to contact urea-rich plasma, lymph or interstitial fluid and to liberate toxic ammonia. If proteolysis cannot be suppressed, the whole chain of events will be blocked.

The in vitro experiments show that Kunitz inhibitor is rapidly degraded by rumen liquor, while the in vitro relatively stable fraction with both trypsin- and chymotrypsin-inhibitor activity may be identical with the Bowman–Birk inhibitor.

In another study we failed to demonstrate the binding of labelled soybean lectin to any microorganism living in the ovine rumen.

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STUDY OF THE MUTAGENIC EFFECT OF REDENTIN ON VARIOUS SPECIES OF ANIMALS

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The mutagenic effect of Redentin was studied on *(i)* chicken embryo cells of Plymouth hens (obtained from eggs laid either by Redentin-treated hens mated with untreated cocks or untreated hens mated with treated cocks); *(ii)* bone-marrow cells of treated Peking ducks; and *(iii)* bone-marrow cells and spermatocytes of treated rabbits. The experimental dose of Redentin induced no chromosome aberrations in any of the examined cells.

Keywords. Rodenticide, chlorophacinone, mutagenicity, hen, Peking duck, rabbit.

Redentin is a cumaroid derivative that has a selective toxic effect on rodents (Bentley, 1972; Kékes-Szabó and Vörösházi, 1978). According to our previous investigations, the experimental dose of 20 mg/kg had no mutagenic effect on the bone-marrow cells (Selypes et al., 1981) and on the spermatocytes (Nehéz et al., 1984) of the mouse. Since this chemical is used in industrialized large-scale agriculture (Berencsi et al., 1978), wild animals living in fields and meadows, and even domestic animals occasionally staying there, are exposed to contamination. For this reason, it was considered expedient from the point of view of environmental hygiene to study the possible mutagenic effect of Redentin on further species of animals.

Materials and methods

The following animals were used:

Domestic fowl: hens and cocks of the Plymouth strain, and Peking ducks.

Wild mammal: rabbit *(Oryctolagus cuniculus)*.

(I) *Plymouth hen.* First a group of ten hens was treated through a gastric cannula per os with 100 mg/kg Redentin and mated with untreated cocks; then two cocks were treated per os with 100 mg/kg Redentin and mated with untreated hens. This dose is a quarter of the acute p. o. LD_{50} (Berencsi et al., 1978). For control the eggs of 10 untreated hens were used, laid after the hens had been mated with untreated cocks.

The eggs laid on days 3 to 8 after treatment were incubated at 38 °C and 60% relative humidity. On the 7th day of incubation the embryos were removed and homogenized in a grinding mortar containing Parker solution

(Difco TC 199). Then culture was made at 37 °C for 30 min while the mitosis was hindered with a single dose (3 μg/10 ml medium) of Vinblastin. Hypotonization was carried out with a 0.075 M KCl solution for 20 min. Fixation was made with a mixture of methanol–acetic acid at the rate of 3:1. The preparations were stained with Giemsa solution. The chromosome analysis was made after Stock and Bunch (1982).

(II) *Peking duck.* For the chromosome examination of the bone-marrow, four ducks, with an average weight of 2700 g, were given, with a gastric cannula, a single dose of 50 mg/kg of Redentin, which corresponds to a quarter of the acute p. o. LD_{50} for ducks (Berencsi et al., 1978). Following the treatment (24 and 48 h), bone-marrow obtained from the tibia was homogenized in Parker solution, then cultured at 37 °C for an hour. The preparation was carried out as described above. From the bone-marrow cells of each of the two treated and two untreated birds 50 samples of mitosis were evaluated according to the method of Stock and Bunch (1982).

(III) *Rabbit.* Five male animals with an average weight of 2400 g were treated per os through a gastric cannula with 20 mg/kg Redentin, a quarter of the acute p. o. LD_{50} for rabbits (Kékes-Szabó and Berencsi, 1980), then 48 h after giving this single dose, the preparation of the bone-marrow and the examination of the spermatocytes were performed as stated above. The chromosome analysis was made after standard karyotype of the laboratory rabbit (1981).

The samples of mitosis were evaluated under microscope, magnified by 1600 in each case. Statistical significance was computed by the Fisher test (Delaunois, 1973).

Results and discussion

The data obtained for eggs and chicken embryos are shown in Table I.

Table I

Examined eggs and embryos

Group	Treatment p.o.	Number of eggs examined	Number of infertile eggs	Number of dead embryos	Number of eye aberrations
I	Hen: Redentin 100 mg/kg	16	3	2	—
	Cock: Ø				
II	Cock: Redentin 100 mg/kg	30	7	2	1
	Hen: Ø				
Control	Ø	16	3	1	1

Ø: no treatment

Three of the 16 eggs obtained from the control group were not fertilized and 1 of the embryos was dead while 1 embryo had three eyes. In the group of the treated hens, 3 of the examined 16 eggs were not fertilized and 2 of the embryos were dead. In the group of the treated cocks that mated with the untreated hens, 7 of the 30 eggs were not fertilized, 1 embryo was dead and 1 had only one eye.

The data obtained from the chromosome examinations of the chicken embryos are shown in Table II.

Table II

Results of chromosome examinations of chicken embryos

Group	Treatment p.o.	Number of embryos	Number of cells examined	Cells with aberrations	Aberrations					
					numerical	gap	isogap	chromosome exchange	deletion	pulverization
I	Hen: Redentin 100 mg/kg	6	120	18	6	7	3	—	2	—
	Cock: Ø									
II	Cock: Redentin 100 mg/kg	8	160	16	7	7	—	1	2	1
	Hen: Ø									
Control	Ø	8	160	12	3	9	—	—	—	—

Ø: no treatment

Aberrations were seen in 18 of the 120 mitotic cells examined in the first group, and in 16 of the 160 samples examined in the second group. These were mostly numerical and gap changes and, compared with the control data, not significant.

The effect of Redentin on the bone-marrow cells of the ducks are presented in Tables III and IV.

Table III

Examination of the mutagenic effect of Redentin in the chromosomes of the bone-marrow of ducks 24 hours after treatment

Material	No. of cells examined	No. of cells with aberration	No. of cells with aberrations			
			numerical aberration	gap	break	deletion
Redentin 1×50 mg/kg	50	8	1	1	3	3
Control	50	10	7	1	—	2

Table IV

Examination of the mutagenic effect of Redentin in the chromosomes of the bone-marrow of ducks 48 hours after treatment

Material	No. of cells examined	No. of cells with aberration	No. of cells with aberrations			
			numerical aberration	gap	break	deletion
Redentin 1×50 mg/kg	50	13	3	5	1	4
Control	50	13	4	3	1	5

As the Tables show, from the 50 samples of mitosis 8 aberrations were observed 24 h after the treatment and 13 aberrations 48 h after the treatment in this group. Neither of these changes was significant as compared with the control.

The results obtained by the examination of chromosomes from the treated rabbits' bone-marrow cells are presented in Table V.

Table V

Results of chromosome examinations of bone-marrow cells obtained 48 hours after treating rabbits with 1×20 mg/kg of Redentin

Group	Number of examined cells	Number of cells with aberrations	No. of cells with aberrations				
			numerical aberration	gap	break	acentric fragment	deletion
Treated	250	38	8	11	1	3	17
Control	250	40	17	6	3	6	11

Of the 250 samples of mitosis examined in each of the treated and control groups, nearly the same number (38 and 40, respectively) showed aberrations. In the treated group deletions (17) and gaps (11), while in the control group numerical changes (17) and deletions (11) occurred most frequently. The differences, being nonsignificant, cannot be attributed to the treatment.

In the course of the spermatocyte examinations, only a few univalent chromosomes were observed. The frequency of changes in the spermatocytes was 3.2%. This is within the standard deviation of spontaneous aberrations.

In conclusion, Redentin® at the applied dosage had no mutagenic effect on the various animals used in the given experimental circumstances.

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BOOK REVIEW

Handbook of Cryobiology. Author: Tsvetan Tsvetkov, Head of the Central Research Laboratory of Cryobiology and Lyophilisation, Sofia, Bulgaria. Ed. by ZAMIZDAT, Sofia, Bulgaria. 316 pages, 144 figures, 121 tables, 354 references.

The present handbook is dedicated to a new field of science — cryobiology — a field of enormous emotional charge and practical importance. In the last decades cryobiology made fast progress due to recent results of cryophysics and cryogenics. The Handbook considers the thermodynamical foundations of cryogenics, properties of working substances and the basic cryogenic machines, applied in cryobiology practice. Fundamental physico-chemical ideas of water structure and its properties in various solutions, biosystems and food products are presented. Low-temperature crystallization of water in biomaterials is discussed together with the fundamental types of cryoprotectors used for cryopreservation. Basic cryoinstruments and apparatuses applied in medicine, in particular in surgery, are also accounted for. The problem of freeze-drying of biomaterials and the machines for its realization — vacuum freeze-driers — is fully enlightened. The fundamental concepts and terminology in Cryobiology are listed. The Handbook will be of total use for the broadest circle of specialists, working in the field of low-temperature preservation of biomaterials.

R. Lásztity

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NEWS

Forthcoming conferences

20th International Symposion on the History of Veterinary Medicine

May 08 to 12, 1985, in Hannover, Federal Republic of Germany

The World Association for the History of Veterinary Medicine and the Section History of Veterinary Medicine of the German Veterinary Association arrange their 20th International Symposion on the History of Veterinary Medicine from May 08 to 12, 1985, in Hannover (Federal Republic of Germany).

All interested people — also non-veterinarians — are cordially invited to take part. Those who have not yet joined these meetings will please contact the secretary's office of the World Association for the History of Veterinary Medicine: Tierärztliche Hochschule Hannover, Bischofsholer Damm 15, D-3000 Hannover 1 (Federal Republic of Germany), Tel. (0511) 856 503, Telex 9 22034 tiho d. The symposion material will be sent out well-timed. If desired an application form for membership in the World Association will be sent.

Hitherto unpublished lectures for the topics "The Dog in Veterinary Medicine until the 19th Century" and "The Affairs of Veterinary Museums" as well as for free historical themes, which will not be estimated different for the purposes of the symposion, can be announced compulsorily to secretary's office not later than December 31, 1984, by giving the title in the language of the lecture, and in English. Between 10 and 30 minutes will be available to each lecturer. Announcing the lectures their duration should be mentioned. Official languages are English, French, German and Spanish.

5th International Congress on Animal Hygiene

Date: from Tuesday, 10. 9. 1985 to Friday, 13. 9. 1985
Place: Tierärztliche Hochschule Hannover, Federal Republic of Germany.
Organized by the International Society for Animal Hygiene, in cooperation with the Workshop of Animal Hygiene of the German Veterinary Society.
Languages of the congress: English and German with simultaneous interpretation; Russian without simultaneous interpretation.
Programme: Scientific short lectures (10 minutes each) in three sections:
A) Management and production hygiene
B) Hygienic problems of animal wastage, including manures
C) Free themes from the field of animal hygiene
Titles and abstracts of lectures should be sent to the Congress Secretariate before 28. 2. 1985.
Congress Secretariate: Sekretariat des V. Internationalen Kongresses für Tierhygiene, Professor Dr. Hans G. Hilliger, Institut für Tierhygiene der Tierärztlichen Hochschule Hannover, Bünteweg 17 P, D-3000 Hannover 71, Bundesrepublik Deutschland.

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