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THE SUPPOSED INVOLVEMENT OF DOGS CARRYING *BRUCELLA SUIS* IN THE SPREAD OF SWINE BRUCELLOSIS

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Reappearance of brucellosis in a pig production unit re-stocked with brucellosis-free sows and boars after liquidation of the original infected stock is reported. Epizootiological investigation and laboratory tests revealed that a watchdog, which had been kept in the unit for several years and had had contact with the liquidated herd, was *Brucella* carrier and acted as source of the infection. The dog was serologically positive, and a *Brucella suis* type-2 strain, identical with that isolated from a pig fetus aborted during the outbreak of brucellosis in the sow herd, was isolated from its organ homogenate in guinea pigs.

Brucella canis infection, responsible for abortion in bitches, was originally described by Carmichael and Bruner in 1968. In the meantime much information has been accumulated on the epizootiological and immunological aspects of the disease.

The epizootiology of other canine *Brucella* infections has been little studied. Prior (1976) presented bacteriological evidence that symptomless dogs carrying *B. abortus* served as reservoirs of the agent. Bicknell and Bell (1979) isolated *B. abortus* from the urine of a house-dog, which had acquired the infection probably from a nearby cattle herd affected by brucellosis. Harrington and Brown (1976) reported *B. suis* type-1 infection in a dog without pursuing the case history.

We, too, observed *B. suis* infection in a healthy-appearing male dog, and describe our pertinent investigations, which suggest a mediator role of the dog in swine brucellosis, in this paper.

Case history

A large pig unit was re-stocked with a new breeding herd after liquidation of the original infected population. For re-stocking, 200 gilts were procured from a brucellosis-free herd, and 6 breeding boars from another "clean" unit.

Before introduction into the unit, the gilts had been quarantined in distant premises for 35 days and inseminated and examined serologically during that period. The results of serological screening were negative, and the gilts were established in the unit which had been evacuated for 3 months, and thoroughly cleaned and disinfected.

No signs indicative of brucellosis were observed during the first pregnancy of the gilts. Serological screening, performed after the first farrowing, showed them to be free from brucellosis, leptospirosis, and Aujeszky's disease.

The 6 breeding boars were sold after the first mating cycle to a brucellosis-free pig farm. All were clinically healthy, and serologically negative for brucellosis at sale and for one subsequent year.

For the second mating cycle, 7 boars were procured from the herd of origin of the sows; that herd had been free from brucellosis for several preceding years. The newly-procured boars were used for natural breeding. During the second breeding cycle, 3 sows aborted their fetuses between days 103 and 106 of gestation. Two of them had 80 IU/ml anti-brucella agglutinin in the serum, and all 3 had complement-fixing (CF) antibodies. Eleven days after the abortions, we demonstrated 268 IU/ml anti-brucella agglutinin and specific CF antibodies in the serum of one of the 7 boars, which, however, did not show any clinical indication of brucellosis. Of 15 examined sows, 12 had CF antibodies to brucella and 3 had agglutinins. Fifty-three days after the first abortions, some sows aborted again, and *Brucella suis* type-2 antibodies were demonstrated in the miscarried fetuses.

Epizootiological investigations on the source of infection covered 4 draught horses used earlier in the unit for fodder transport, a 6-year-old male watchdog kept in the unit for several years, the village boar sire, and all pigs privately owned by the farm attendants for backyard rearing (further on referred to as backyard pigs).

The horses and the village boar sire gave negative results in both the tube agglutination and CF test. Provocation of the backyard pigs with brucergene also failed, but the dog showed positive CF reaction, up to 1:80 serum dilution.

Materials and methods

Two urine samples were taken from the watchdog, a clinically healthy male mongrel, at an interval of 2 weeks. The samples were centrifuged at 3000 r.p.m. for 20 min, samples from the sediment were transferred to 5% bovine blood agar and Difco tryptose agar, and were incubated at 37 °C in an atmosphere containing 10% CO_2. The remaining urine sediment was suspended in saline, and 2 guinea pigs, weighing 300 g, were inoculated subcutaneously with 1 ml of the suspension (Alton and Jones, 1967).

The dog was killed, and after examination for gross lesions, 1 g specimens taken from the spleen, liver, testicles, epididymides and pelvic lymph nodes were suspended by grinding in 10 ml saline solution. The pooled organ suspension was centrifuged, and the sediment was used for culturing and inoculation of guinea pigs as above.

One of the two guinea pigs inoculated with urine and one of those inoculated with the organ suspension was killed at 8 weeks, the other at 87 days, after inoculation. The carcases were examined for gross lesions, and spleen suspensions of each were used for brucella isolation as above.

To determine the genus of the isolates we performed the following tests: indole, urea, catalase, oxidase production, citrate utilization, methyl red and Voges–Proskauer tests, nitrate reduction, glucose oxidation and fermentation, and test for motility, all as described in the manual of Cowan and Steel (1975).

The colonies of the isolates were tested for S and R phase, CO_2 requirement, H_2S formation, growth in the presence of basic fuchsin (1:50,000; 1:100,000) and thionine (1:25,000; 1:50,000; 1:100,000), agglutination with monospecific brucella A and M sera as described by Morgan and Gower (1966), and for agglutination with R serum according to Skinner and Lovelock (1979).

Results

No gross lesions were found at post-mortem examination of the dog. *Brucella* grew out neither on streak plates inoculated with the urine sediment, nor on those inoculated with the mixed organ suspension.

The 2 guinea pigs inoculated with urine sediment showed no gross indications of brucellosis at post-mortem examination, and attempts to isolate *Brucella* from their spleens also failed.

However, the guinea pigs inoculated with the dog's organ suspensions showed at autopsy swelling of the spleen and granulation of its surface; the granules were coarse, greyish-white, and did not rise above the surface. The liver, too, was slightly swollen owing to serous oedema, and there were a few yellowish, non-prominent pin-prick foci on its surface.

On the streak plates inoculated with spleen suspensions on blood agar and tryptose agar, Gram-negative, Köster-positive coccoid bacteria, forming greyish, transparent, honey-drop-like, prominent "S" colonies the size of a pinhead, grew out after incubation for 5 days in aerobic conditions.

The isolate showed oxidase, catalase and urease activity, but did not produce H_2S. It neither decomposed nor oxidized glucose, did not produce indole from tryptophan and reacted negatively in the methyl red and Voges–Proskauer tests. It reduced nitrate, but did not utilize citrate, and showed no motility. It did not form colonies on media containing basic fuchsin or 1:25,000 thionine, but grew well in the presence of higher dilutions (1:50,000; 1:100,000) of thionine. It was not agglutinated by the *Brucella* M monospecific type serum. On the basis of these criteria, the isolate was identified as a ***Brucella suis*** type-2 strain.

The *B. suis* type-2 strains isolated from the aborted pig fetuses agreed in every respect with those isolated from the dog through a passage in guinea pig.

Discussion

In a sow herd originating from a brucellosis-free stock, we observed abortions due to *Brucella suis* type-2 during the second breeding cycle. Serological screening showed that the sows which had aborted, and one of the 7 boars used for breeding, were infected by *Brucella*.

Since the first pregnancy and the first farrowing of the sows had been uncomplicated, and post-farrowing serological screening for *Brucella* had had negative results, we postulated that the infection had taken place during the second pregnancy. Carriership could be ruled out with certainty, for the boars used for the first service, and those used for natural breeding in the second cycle also had originated from a brucellosis-free herd. We therefore looked for the source of infection in the unit itself.

The draught horses used in the unit for fodder transport, the village boar sire, and the privately owned backyard pigs of the farm attendants all proved to be serologically negative for brucellosis. However, the watchdog of the unit, a 6-year-old male mongrel, was serologically positive, and the strains isolated from its organs by experimental infection of guinea pigs represented the same *B. suis* biotype as the isolates obtained from the aborted pig fetuses.

We thus concluded that the source of *Brucella* infection in the originally brucellosis-free herd was in all probability the watchdog, which had presumably acquired the infection from the previous sow herd, liquidated on account of brucellosis.

Like other investigators, we found that the *B. suis* infection caused neither symptoms, nor gross lesions in the dog. Unlike others, we failed to isolate *Brucella* from the urine of the dog, but this does not, of course, exclude shedding of the pathogen with dog urine in certain periods.

The fact that isolation of *Brucella* from the pooled organ suspension of the dog was successful only after a passage in experimental animal, indicates that only few viable cells of *B. suis* may have been present in the organs of the carrier.

This accords well with our assumption that the dog had acquired the infection from the pig herd liquidated 2 years earlier.

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ANTIBACTERIAL EFFECT OF VOLATILE FATTY ACIDS ON ENTEROBACTERIACEAE IN THE LARGE INTESTINE

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(Received January 25, 1982)

The volatile fatty acid- (VFA-) associated antibacterial activity of the large-intestinal contents of healthy rabbits and swine was studied in vitro. Strains of bacteria (*Salmonella, Shigella, E. coli, Proteus*) belonging to Enterobacteriaceae were used. VFA concentrations (70–120 mmol/kg) of intestinal contents inhibited bacterial multiplication within the pH range of 6.0–6.5, but this inhibitor activity disappeared above pH 6.8. The 55 species of bacteria studied in vitro showed identical sensitivity to the antibacterial activity of VFA.

In large-intestinal contents of rabbits and swine the intensity of antibacterial activity was similar at identical pH values. According to results obtained by studies on faecal samples of humans, an antibacterial action of VFA in the large intestine of humans can also be postulated. The role of VFA-associated antibacterial activity of intestinal contents in the pathomechanism of enteric infections is discussed.

During earlier investigations we have observed that VFA in the caecal contents of healthy rabbits exert an inhibitor activity on the multiplication of *E. coli*. Caecal contents of diarrhoeal rabbits exhibit decreased or no antibacterial activity. This leads to an excessive multiplication of *E. coli* bacteria resulting in the death of rabbits (Prohászka, 1980; Prohászka and Baron, 1981). This observation raises the question whether the antibacterial effect of VFA also plays a role in the pathomechanisms of diseases caused by other bacteria, and whether intestinal contents of other species of animals also show the above phenomenon. The present investigations were aimed at elucidating these questions.

Materials and methods

Species of bacteria belonging to the Enterobacteriaceae family were studied. The strains used and their numbers are shown in Table I. Some of the strains were maintained in the laboratory. Among the *E. coli* strains there were ones belonging to human enteropathogenic serogroups (026: K60/B/; 055: K59/B/; 0125: K70/B/ and those of serogroups pathogenic to swine (06: K53/L/; 0138: K81; 0139: K82; 0141: K88; 0149: K88, 91). Other strains were isolated from human faeces 2–4 weeks before the experiments (fresh isolates). These partly derived from patients exhibiting clinical symptoms

(diarrhoea), partly from symptomless individuals. We do not consider necessary to indicate the origin and to give detailed characterization of the strains since they behaved uniformly during the experiments, and no distinction could be drawn between them.

Intestinal contents derived from 2 species (rabbits and swine) were studied. The samples were obtained from healthy individuals showing no symptoms of enteric disease.

Table I

Strains of bacteria used in the experiments

	n
Salmonella typhimurium	4
Salmonella enteritidis	2
Salmonella oranienburg	1
Salmonella derby	1
Salmonella bareilly	1
Shigella sonnei	6
Shigella flexneri	6
Klebsiella pneumoniae	2
Klebsiella aerogenes	2
Enterobacter cloacae	2
Escherichia coli	22
Proteus vulgaris	2
Proteus mirabilis	2
Proteus morganii	1
Proteus rettgeri	1
	55

The rabbits (New Zealand rabbits of 3–4 months of age) received a conventional diet (18% crude protein, 10% crude fibre), they were bled and caecal contents were removed. The swine (meat-type hybrids) of 4–5 months of age received a conventional swine diet (17% crude protein and 3% crude fibre). From exsanguinated swine the large-intestinal contents were removed. The samples derived from the two species were processed further in an identical way. The intestinal contents were autoclaved for 30 min at 120 °C to destroy the original intestinal flora. Twenty g quantities of the autoclaved intestinal contents were placed in sterile glass flasks, and the pH of samples was adjusted to the values shown in Fig. 1 by the addition of 1 N NaOH or 1 N HCl. To these samples bacterial suspensions were added so as to adjust the preincubation bacterial counts to 10^5/g. The culture flasks were sealed with parafilm

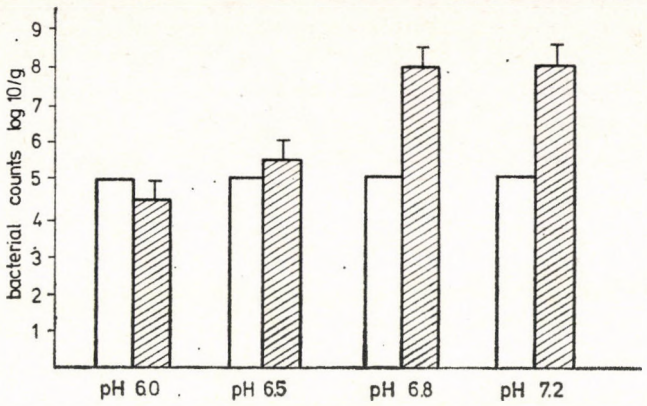

Fig. 1. Antibacterial effect of volatile fatty acids in large-intestinal contents of rabbits and swine on Enterobacteriaceae at different pH values. White columns, bacterial counts before incubation; striated columns, bacterial counts after incubation

(parafilm-M, American Can Company) and incubated for 20 h at 37 °C. Then, the bacterial count was determined for each culture. The water content of the samples was adjusted to a value similar to that of the original intestinal contents (70–72%).

From humans, faecal samples were collected and examined as described above.

Bacteriological studies

Bacterial suspensions were prepared from 24 h cultures with 0.9% NaCl solution. For determining bacterial counts, serial dilutions were prepared in PBS. Media containing agar with 5% sheep erythrocytes and lactose were used for bacteriological studies.

The pH of samples was determined in an electric pH meter (Radiometer Copenhagen 28), and VFA concentrations with a Hewlett–Packard 5720 A Gas Chromatograph.

For gas chromatographic analysis, samples were diluted 1:10 in distilled water, and the pH values were adjusted to 2 by addition of 50% H_2SO_4. The suspension was then shaken for 30 min and centrifuged. To 0.5 ml of the supernatant 0.5 ml 30% formic acid was added, and 2 μl of the mixture was used for gas chromatographic analysis, under the following conditions: carrier gas N_2 40 ml/min; hydrogen 40 ml/min; air 250 ml/min. Column packing: Chromosorb-101, mesh 100–120 (Carlo–Erba). Column temperature: 160 °C. Injector temperature: 250 °C. Detector temperature: 250 °C.

The total detectable quantity of volatile fatty acids (VFA) represents the sum of 3 VFA components (acetic acid, C_2; propionic acid, C_3; butyric acid, C_4), and it was expressed in terms of mmol/kg of wet weight.

Results

Results are shown in Table II. From rabbits, caecal contents were examined since these represent the major part of the total intestinal volume. From swine, colon contents were examined since caecal contents of swine are negligible.

Faecal samples were examined in order to elucidate the changes occurring during rectal passage, and to show how to estimate data for the caecal contents on the basis of faecal analysis. Data on stomach and small-intestinal contents are not shown in Table II, since these had so low VFA concentrations (6–14 mmol/kg) that antibacterial effect of VFA cannot be reckoned with in these portions of the digestive tract.

The large-intestinal contents of swine had by 50% higher VFA concentration than those of rabbits, and a similar difference was demonstrated between the VFA concentrations of the faeces. There was a difference also in the component distribution of VFA between rabbits and swine. In rabbits only traces of propionic acid were detectable, while in swine this fatty acid represented 20–30% of the total VFA.

Large-intestinal contents of rabbits showed lower pHs than those of swine. The acid character of large-intestinal contents gradually ceased during excretion, the pH usually became mildly basic. This change can be observed in both species studied.

Table II

E. coli counts, pH values and volatile fatty acid (VFA) concentration in intestinal contents of rabbits, swine and humans

	E. coli count $\log_{10}$/g			pH			VFA mmol/kg			C_2 %	C_3 %	C_4 %
	$\bar{x}$	SD	n	$\bar{x}$	SD	n	$\bar{x}$	SD	n	$\bar{x}$	$\bar{x}$	$\bar{x}$
Rabbit caecum	3.5	1.5	10	5.9	0.15	10	76	12	10	80	8	12
faeces	3.8	0.7	10	7.2	0.22	10	45	20	10	86	2	12
Swine colon	7.2	1.2	10	6.5	0.30	10	112	19	10	60	32	8
faeces	7.8	0.7	10	7.1	0.15	10	78	16	10	67	20	13
Human faeces	7.0	1.2	10	7.0	0.32	10	84	12	10	59	30	11

In Table II, only *E. coli* counts are shown since the number of *E. coli* bacteria is the most reliable indicator of the intensity of the antibacterial effect exerted by VFA in the large intestine. Coli counts in large-intestinal contents and faeces of healthy rabbits were approximately by 3 $\log_{10}$ lower than those for swine, a fact indicating more intensive antibacterial activity in the large intestine of rabbits.

Data obtained with human faeces show that VFA content and composition, pH and coli numbers in the large intestine of humans are similar to those

for swine. On the basis of these results an antibacterial activity similar to that found in swine must be expected in humans.

Figure 1 illustrates antibacterial activity of VFA in large-intestinal contents of rabbits and swine. When selecting samples of large-intestinal contents, we chose samples with VFA content characteristic of the species, both quantitatively and qualitatively. Thus, VFA contents of samples derived from rabbits and swine were 70–80 mmol/kg and 100–120 mmol/kg, respectively.

During earlier investigations (Prohászka, 1980), we found that the pH of samples influenced the antibacterial activity of VFA. Therefore, samples derived from the two species were always compared at identical pH values adjusted by us. In previous experiments we found that in healthy and diarrhoeic individuals of the two species pH values of large-intestinal contents ranged mostly between 5.7 and 7.4. Therefore, we chose pH values within this range (data shown in Table II).

The antibacterial activity of caecal contents of rabbits and swine was tested with 55 strains of bacteria. Results of these experiments can be drawn together in one figure since all strains behaved in a practically identical way, and samples derived from the two species also exhibited a similar antibacterial activity at identical pH values.

At pH 6.0 the numbers of all bacteria remained constant or decreased. At pH 6.5 the multiplication of bacteria was insignificant, while at pH 6.8 it reached the degree shown at more basic (pH 7.2) values. The number of bacteria developed during the 20 h incubation period was generally consistent with *E. coli* counts observed in animals naturally affected by *E. coli* diarrhoea.

The antibacterial activity of large-intestinal contents was of similar intensity to all strains of bacteria studied. *E. coli*, *S. typhimurium* and *Proteus vulgaris* strains cultured in samples containing VFA through 10 passages failed to develop resistance.

Discussion

Effects of VFA on the intestinal flora were first studied in experiments conducted with gnotobiotic mice and rats (Berg and Owens, 1979; Byrne and Dankert, 1979; Lee and Gemmel, 1972; Leegwater et al., 1974; Lewison, 1973; and Meynell, 1963). There are insufficient data on the role of the antibacterial activity of VFA in enteric diseases of domestic animals and man. Prohászka and Baron (1981) pointed out that in the pathogenesis of diarrhoea showing mass incidence among weaned rabbits and considered to be associated with *E. coli* infection the primary aetiological factor is an impaired transport of ions due to inappropriate feeding. This disorder leads to a decrease in the antibacterial activity of VFA in the large intestine, allowing excessive secondary multiplication of *E. coli* bacteria in the intestinal tract. This observation

suggests that the above mechanism may play a role also in enteric diseases of other species. The possibility of this pathomechanism was studied in two species (rabbits and swine) in which the occurrence of enteric diseases is frequent.

In the large intestine of both species VFA occur in such quantities and quality that their antibacterial activity must be reckoned with in healthy individuals. In previous experiments (Prohászka, 1980) we found that at pH values occurring in the large intestine at least 50 mmol/kg or more VFA are needed for the inhibition of *E. coli* multiplication. Our results obtained so far indicated that the large-intestinal contents of rabbits and swine always contained the amount of VFA required for antibacterial activity.

The present results indicate that the VFA in the large intestine have an inhibitor activity to not only *E. coli* but also other members of Enterobacteriaceae (*Salmonella*, *Shigella*, *Proteus*, *Klebsiella*). From the experimental results it appears that the intensity of the in vitro antibacterial activity is similar in the samples derived from rabbits and swine, and all members of the Enterobacteriaceae family are practically equally sensitive to this activity.

From studies on human faeces we can come to the conclusion that the VFA concentration present in the large intestine of humans is similar to that found in the large intestine of swine. Presumably, the antibacterial effect of the human VFA is also similar to that of the VFA of swine. *E. coli* counts found in the faeces of healthy humans and healthy swine (10^6–10^8/g in both species) also indicate similar intensities of activity. On the other hand, significantly lower *E. coli* counts can be found in the large intestine of rabbits, indicating that here the antibacterial activity of VFA considerably exceeds that observed in the other two species. The biochemical explanation of this phenomenon can be found by comparing the pH values of large-intestinal contents. The large-intestinal contents of rabbits had generally by 0.4–0.5 degrees lower pH values than those of swine. At lower pH values the proportion of non-dissociated VFA molecules which account for a greater bacteriocidal action, increases.

Parallel with the rise of large-intestinal pH, the proportion of non-dissociated VFA molecules gradually decreases. At pH 6.8 these molecules are present in so small quantities that no antibacterial activity can be detected. This sensitivity to pH calls our attention to the fact that in spontaneously occurring cases not the decrease in the quantity of VFA, but the increased ratio of dissociated to non-dissociated molecules due to the rise of pH, accounts for the reduced antibacterial activity. The pH of the large intestine depends on numerous mechanisms regulating the transport of ions (Field et al., 1980). Disorders of these mechanisms may easily lead to increased pH values.

From literary data, an enteric syndrome is known which is accompanied by a decrease of VFA concentrations instead of the rise of pH. This enteric

syndrome has been observed as an undesirable side effect (diarrhoea) during treatments with certain antibiotics, usually after treatment with ampicillin, lincomycin and clindamycin. These observations primarily apply to humans (Benner and Tellman, 1970; Fesce et al., 1978; Sweeney and Sheehan, 1979; and Totten et al., 1978), although ampicillin-induced diarrhoea has already been observed also in rabbits (Morisse et al., 1979).

According to the results of Fesce et al. (1978), Imai and Morishita (1978) and our own observations, the pathomechanism of diarrhoea associated with antibiotic therapy is postulated to be the following: during antibiotic therapy VFA-producing anaerobic bacteria (mainly *Bacteroides*) decrease in number, consequently, the above antibacterial activity disappears, and pathogenic bacteria resistant to antibiotics multiply excessively. The excessive multiplication of bacteria leads to clinically apparent enteritis. This very observation emphasizes the significance of the antibacterial activity of VFA, since it seems that resistance to VFA fails to develop, VFA being self-limiting substances of excessive multiplication of bacteria (Landwall and Holme, 1977).

The intensity of antibacterial activity in the large intestine is determined by two factors: the quantity of VFA produced by anaerobic bacteria (*Bacteroides*, *Fusobacterium*), and the pH of the intestinal contents, regulated by the enteric ion transport of the organism. A harmonious interaction of the macro- and microorganisms is necessary for the optimum functioning of this genuine antibacterial defence mechanism.

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THE IMPORTANCE OF LIVER LESIONS IN THE PATHOGENESIS OF DUCK PLAGUE

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Aquatic birds diseased with acute duck plague (viral enteritis) usually also develop an acute liver distrophy. The direct responsibility of the herpesvirus agent of duck plague for the liver involvement has been substantiated by morphological evidence. Replicating forms of virus have been demonstrated mainly in the hepatocytes showing degeneration and intranuclear inclusion formation. Since the greater part of the birds with acute duck plague also develops liver dystrophy, complementary diagnostic evidence by electron microscopic detection of the virus in, and isolation of it from, the liver is of immediate interest.

While the acute form of duck plague is developed mainly by day-old and growing aquatic birds, it may take a prolonged (chronic) course in older (growing or adult) birds. The naturally and experimentally infected birds examined in a more advanced stage of the disease showed a subacute or chronic interstitial hepatitis, which may account for sporadic mortality in field conditions.

The diagnostic identification of the chronic forms of duck plague is important from the epizootiological point of view, since the birds surviving the chronic course may become symptomless excretors of the virus and shed it in long term in the flock.

Authors generally agree that duck plague (viral enteritis), a common viral disease of aquatic birds (ducks, geese and swans) takes an acute or hyperacute course, during which the causal agent appears in the blood and organs (Jansen, 1968; Leibovitz, 1971; Proctor, 1975, 1976; Bergmann et al., 1979). As to the liver lesions associated with the condition, authors have described focal hepatocellular necroses, haemorrhages, and type-A intranuclear inclusions of Cowdry (Gailiunas and Dardiri, 1970), but no other change.

In pertinent studies (Vetési et al., 1982) we observed that, against the prevailing concepts, outbreaks of duck plague may occasionally take a prolonged course, and apart from the above liver lesions, certain important morphological changes, not yet described by others, appear in both acute and chronic cases of the disease. The latter changes are as a rule of diagnostic value.

This prompted us to investigate the acute and chronic liver lesions associated with duck plague in great detail.

Materials and methods

Embryonated duck and goose eggs, pre-incubated for 12–14 days, were infected with duck-plague virus isolated from a mallard duck. Fifteen several days old mallards, 15 several days old domestic ducklings, 10 day-old goslings,

on a total 25 growing (4–6 weeks old) mallards and domestic ducks, and 10 adult geese were infected by the oral, subcutaneous or intraperitoneal route, with duck-plague virus, partly with 1:100 dilution of the allantoic fluid obtained from the second egg passage, partly with 1:10 dilution of a liver homogenate obtained from carcases of ducklings infected subcutaneously when one day old.

All day-old mallards, ducklings and goslings, except a few killed on the 2nd day after infection for light and electron microscopic examination, died within 3–6 days. Among the growing mallards, growing ducks and adult geese a few birds died during the first week, another few in the 2nd to 4th week after experimental infection, but the majority either died only after 4–6 weeks in a slightly emaciated state, or survived until killed in the 8th week.

The light and electron microscopic examinations covered, in addition to the experimentally infected birds, organ (above all liver) specimens collected from 20 aquatic birds (mallards and domestic ducks) involved in natural outbreaks of acute or chronic duck plague.

For light microscopic histological and histochemical study, specimens collected from the liver, and occasionally from other organs, were fixed in 8% neutral formalin, SUSA solution or absolute ethanol. The sections prepared from paraffin-embedded or frozen blocks were generally stained with haematoxylin and eosin. Neutral lipids were detected with Sudan black, argentophil and collagen fibres with Gömöri's and Van Gieson's techniques; bilirubin was detected with the Gmelin and Stein reaction, glycogen with the PAS reaction.

For electron microscopic study small liver specimens were fixed in 5% glutaraldehyde then in 1% osmium tetroxide, or in 2% osmium tetroxide buffered according to Pallade, were dehydrated in step-graded ethanol solutions and were embedded in synthetic resin. Semi-thin sections were cut and stained with toluidine blue.

Results

Gross lesions

These were essentially similar in the experimentally infected and naturally diseased birds of all species covered in the study.

The birds which died in the acute stage, or were killed in the premortal stage of the disease (day-old mallards, ducklings, goslings and some growing birds) showed a serous-jellinaceous infiltration of the subcutaneous connective tissue in the thoracic and abdominal regions. Intra-abdominal accumulation of a serous exudation was frequently found, and swelling of the spleen occurred in part of the cases.

The liver was as a rule slightly enlarged, and its external and cut surfaces were of variegated appearance, owing to a mosaic-like array of greyish-white

and greyish-red spots. In most cases petechial haemorrhages were present in subcapsular localization. The birds which died in the acute stage also had lesions in the digestive tract; croupous inflammation of the small intestine, diphtheroid inflammatory involvement of lymph-node groups, and presence of small superficial necroses or of larger, circumscribed pseudomembranaceous sloughs in the cloacal mucosa, were frequently found.

The growing or adult birds which died spontaneously or were killed in a more advanced stage of duck plague generally also had livers of variegated appearance, owing to the presence of a few to many greyish-white spots. The consistency of the liver parenchyma was tough in places. In some cases, the liver was small, flaccid to the touch, and greenish-yellow; in the same cases, the gallbladder was usually dilated and contained a thick, greenish-black bile. Necrotic lesions and pseudomembranaceous sloughs were often found in the oesophageal mucosa of those birds which died in later stage of duck plague. Most of the male birds exhibited swelling and prolapse of the penis, and had superficial necroses or pseudomembranaceous sloughs on the mucous membrane of the male organ.

Light microscopic and ultrastructural changes

In liver sections obtained from birds died 3–4 days after infection staining with haematoxylin-eosin or toluidine blue revealed hepatocellular necroses involving one to a few cells in various hepatolobular regions. The damaged cells generally had a vacuolized cytoplasm, with lipid droplets enclosed in part of the vacuoles. Minor to major haemorrhages were seen in some hepatolobular regions. Part of the damaged cells and in places some sinusoidal endothelial and Kupffer's cells enclosed type-A intranuclear inclusions of Cowdry. The hepatocellular necroses were confluent over larger hepatolobular areas or several lobules in the liver of those birds which died 5–6 days after infection. In some cases, the necrotic areas were surrounded by infiltrating heterophil granulocytes. Hepatocellular type-A intranuclear inclusions of Cowdry and intralobular haemorrhages were rarely also present in these cases. The hepatolobular sinusoids were generally dilated, and congested with red blood cells and serum. Frequently, the spaces of Disse were also dilated, and perivascular infiltrations of serum and heterophil granulocytes appeared in the periportal space.

Ultrastructurally, the acute cases were characterized mainly by various hepatocellular changes. Coagulation necroses, involving one to a few hepatocytes, were often found.

In the early stage of coagulation necrosis, single hepatocytes assumed a starlet shape, became smaller and exhibited an electron density more pronounced than usual. The nucleus and the cytoplasmic organelles were still well-

defined; the mitochondria were swollen and distinctly electron-dense, the smooth-surfaced endoplasmic reticulum was dilated in places, and some cytoplasmic vacuoles made appearance. In a more advanced stage of the coagulation necrosis, hepatocytes still localized in the rows near to intact endothelial cells, but they had become very small and highly electron-dense; their cytoplasms contained vacuoles and necrotic cell organelles and the contours of the nucleus appeared indistinct (Fig. 1). Further to these hepatocellular changes, so-called acidophil bodies, formed by necrotic, detached hepatocytes were frequently also present. Virus-like particles could be detected but rarely, and in small numbers, inside the highly electron-dense hepatocytes (acidophil bodies) representing different stages of coagulation necrosis.

Hepatocellular degeneration with appearance of intranuclear inclusions was observed as early as on the 2nd or 3rd day after infection. Initially few, later more numerous hepatocytes showed a slight enlargement, with considerable swelling and low electron density of the nucleus, and loss of definition of the nuclear membrane structure here and there. The nucleolus assumed a homogeneous appearance, and the nuclear chromatin became condensed along the nuclear membrane. As a rule several virus particles made appearance in the nucleus, among a delicately granular and filamentous chromatin residue (Fig. 2). The virus particles were about 90 nm in diameter, and part of them enclosed a distinctly electron-dense core (Fig. 3). The period of virus synthesis was characterized by low electron density of the cytoplasmic matrix, paucity

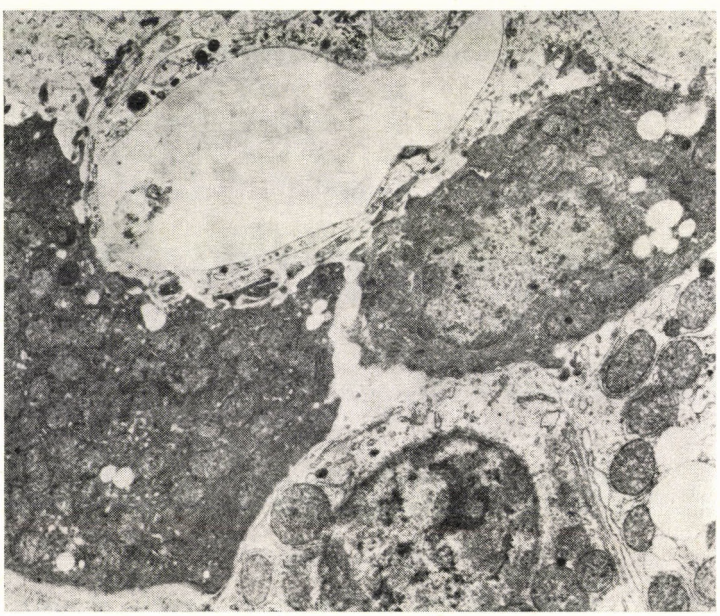

Fig. 1. Hepatocellular coagulation necrosis, ×9000

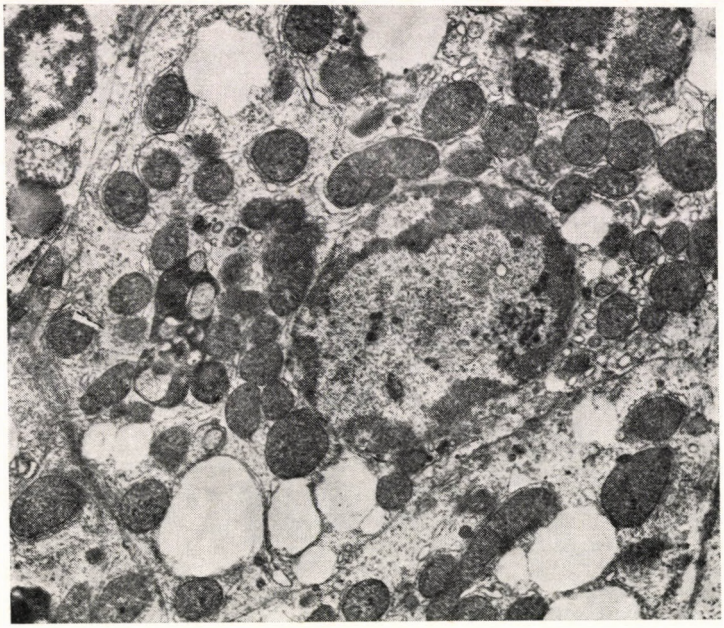

Fig. 2. Virus synthesis in the nucleus of a degenerating hepatocyte, ×10,000

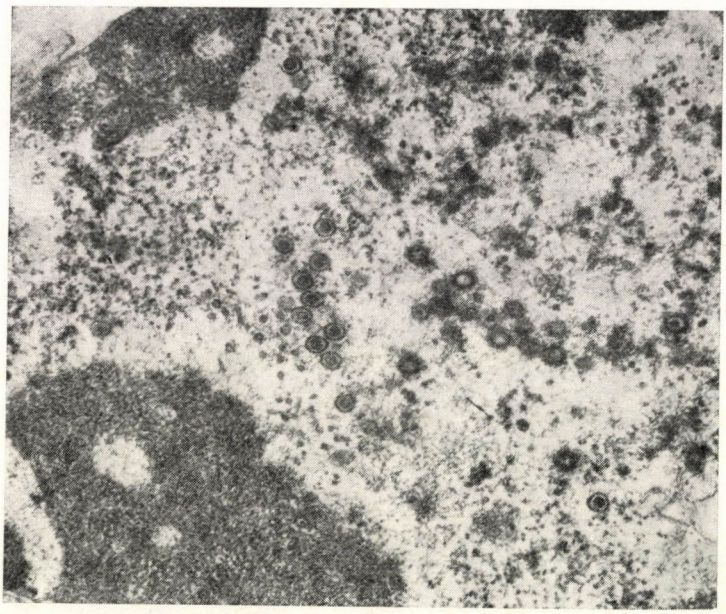

Fig. 3. Intranuclear virus particles with and without a distinct electron-dense core, ×37,000

of intracellular glycogen, and frequent appearance of minor to major lipid droplets between the cell organelles. Most of the mitochondria became swollen and rounded, with electron-dense granules in them. The tubules of the endoplasmic reticulum showed a vesicle-like dilatation in places; the ribosomes became detached and lay freely in the cytoplasm, forming occasionally small groups. The dilated tubules and vesicles of the Golgi complex enclosed an electron-dense substance. Hepatocytes involved in advanced virus synthesis frequently showed duplication of the cytoplasmic and nuclear membranes (Fig. 4), and intranuclear bridge formation by invagination of the nuclear membrane. The virus particles occurring in intra-cytoplasmic localization lay either inside the dilated endoplasmic reticulum, or freely in the matrix, and were enclosed by a thick capsule. The herpesvirus particles endowed with a bilamellar capsule were 160–200 nm in diameter.

Hepatocytes of low electron density also appeared in acute cases of duck plague. These cells were enlarged, and showed the following changes: The hyaloplasm was represented by a delicate helter-skelter network of low electron density. There was paucity and irregular array of endoplasmic reticulum, the mitochondria were swollen and their cristae were indistinct, the nucleus was large, markedly granular, and occasionally a few virus particles were present in it near the nuclear membrane. In some hepatocellular residues of low electron density only remains of the nuclear membrane and chromatin,

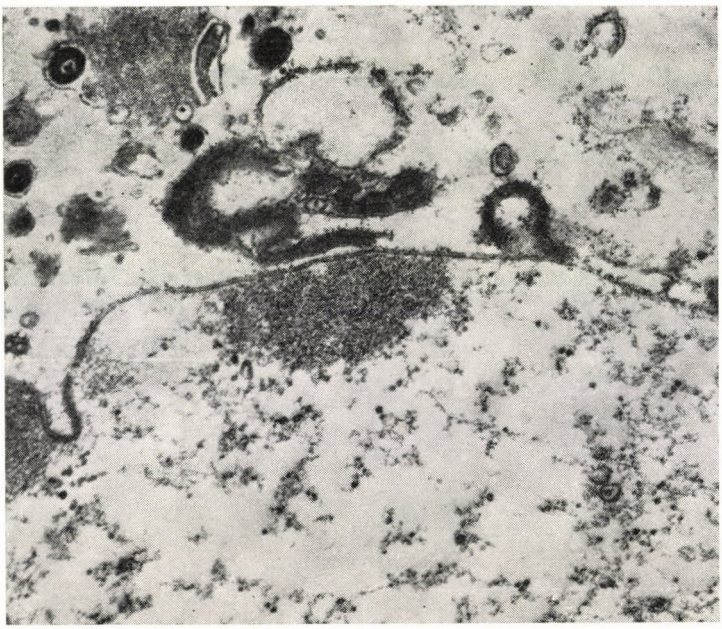

Fig. 4. Reduplication of the cytoplasmic membrane. Virus particles with a bilamellar capsule, × 29,500

and here and there indistinct contours of cytoplasmic organelles could be recognized. In places some sinusoidal endothelial or Kupffer's cells contained virus particles in different stages of development, in intranuclear localizations, with nuclear chromatin condensed along the nuclear membrane. The cytoplasmic changes of the endothelial cells showing virus formation were similar to those seen in parenchymal host cells.

In acute cases, light microscopic examination of the dilated sinusoids revealed presence of acidophil body-like configurations and other cell debris inside the sinusoidal lumen.

Growing and adult birds which died spontaneously or were killed in a more advanced stage of subacute duck plague showed light-microscopically distinct inflammatory cell (above all histio-lymphocytic) infiltrations around interlobular blood and biliary vessels, with occasional extensions into the parenchyma (Fig. 5); indications of fibroblast synthesis could often be seen among the inflammatory cells (Fig. 6). Hepatocellular residues could be recognized here and there in the intralobular areas involved by the cellular reaction. In the non-infiltrated hepatolobular areas the liver cells were preserved and many of them enclosed lipid droplets. The RHS cells of the sinusoidal walls were swollen and showed signs of activation. A few minor hepatocellular necroses, involving single to a few cells, were also present within the lobules.

Electron microscopic examination showed dilatation and irregular shape of bile canaliculi in places, with severe oedema and swelling of the microvilli (Fig. 7); part of the involved microvilli were degenerated. Proliferation and irregular array of the microvilli could also be seen in some bile canaliculi. The dilated lumen enclosed in places a slightly electron-dense, delicately

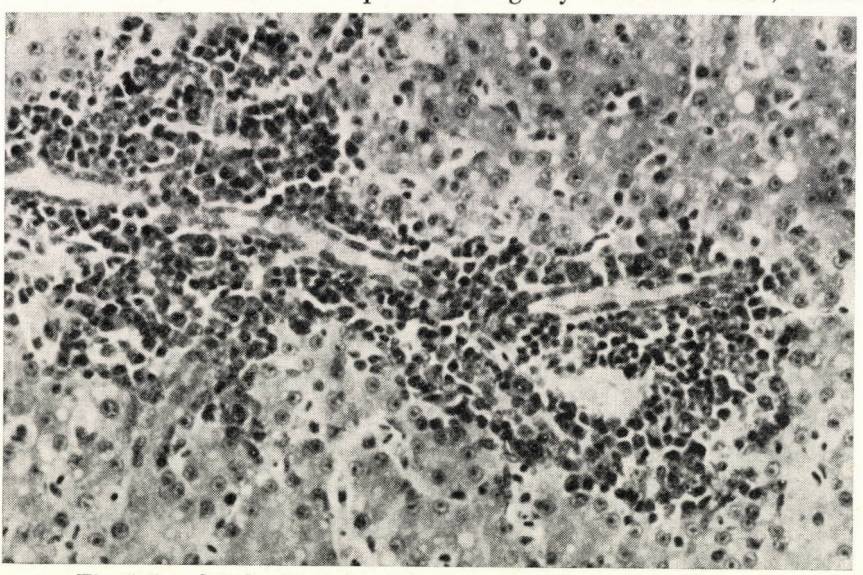

Fig. 5. Interlobular histio-lymphocytic infiltration, ×300; H. and E.

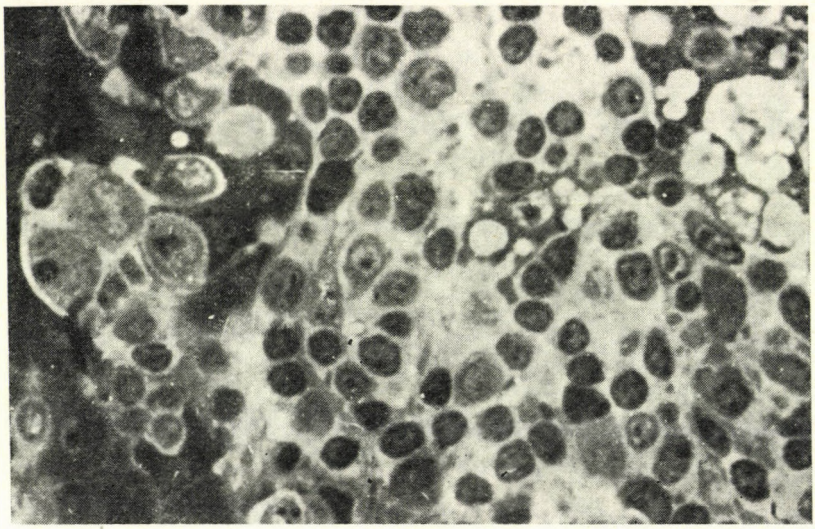

Fig. 6. Fibroblast synthesis and proliferation amongst inter- and intralobular infiltrations of inflammatory cells, ×800; toluidine blue stain

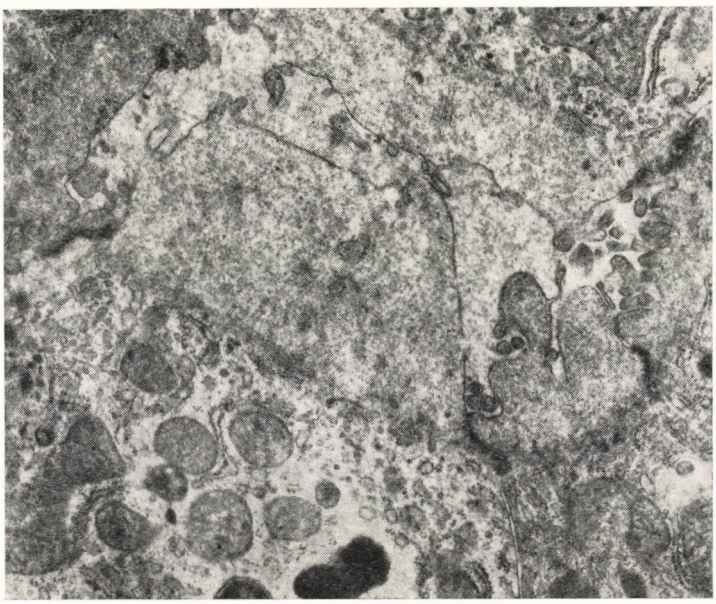

Fig. 7. Oedematous change of the microvilli along the luminal border of the hepatocytes, ×19,000

granular substance; in other places, aggregations of phagolysosomes and pigment granules were seen near biliary vessels (Fig. 8). Some hepatocytes so involved showed a vesicle-like dilatation of the endoplasmic reticulum, and a slight to serious swelling of the mitochondria, with fragmentation of the mitochondrial cristae. Some sinusoids and/or Kupffer's cells enclosed hyaline bodies arisen from necrotic hepatocytes.

In chronic cases, formation of a thick vascular coat, rich in fibroblasts and collagen fibres, was seen in the light microscope around interlobular blood and biliary vessels (Fig. 9); in the experimentally infected cases, sharp delineation of the hepatolobular structure was additionally found. The newly formed interlobular septa were in several places infiltrated by histio-lymphocytes; many infiltrating lymphocytes enclosed electron-dense pigment granules. The cytoplasm of the biliary duct epithelia contained clumps of bile pigment and highly electron-dense granules, and the endoplasmic reticulum showed a vesicle-like dilatation in places. Hepatocellular residues were seen here and there near the thickened interlobular septa.

Electron microscopic examination failed to detect virus particles in preparations originating from 2 or 3 different hepatic regions. Light microscopy, however, revealed type-A intranuclear inclusions of Cowdry, which are indicative of the presence of the virus, in hepatocytes adjoining interlobular vessels, and, occasionally, also in proliferating RHS cells (Fig. 10). Specific virus-neutralizing antibodies were detected in all chronic cases examined.

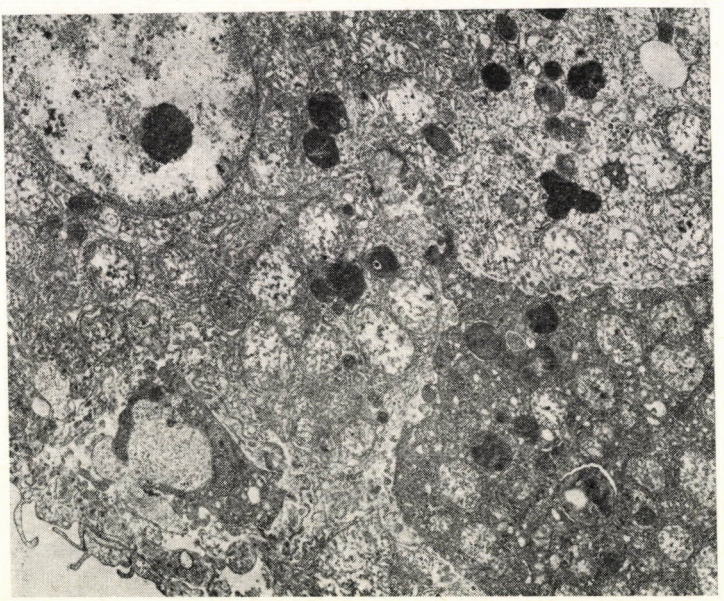

Fig. 8. Bile pigment granules at the luminal side of hepatocytes, ×9000

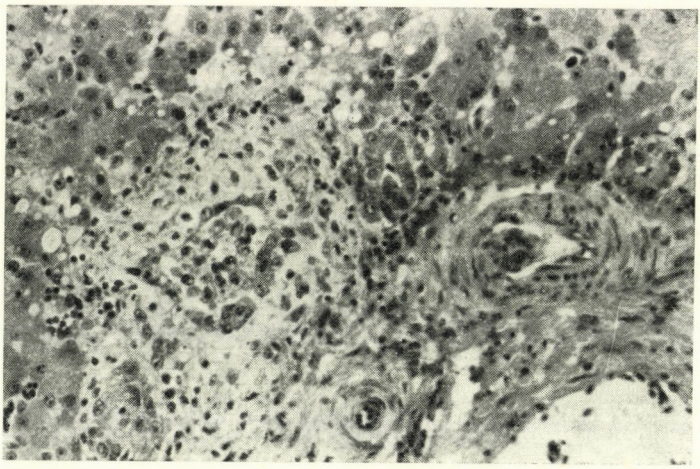

Fig. 9. Chronic duck plague. Formation of vascular coat, rich in collagen fibres, around interlobular blood and biliary vessels, ×300; H. and E.

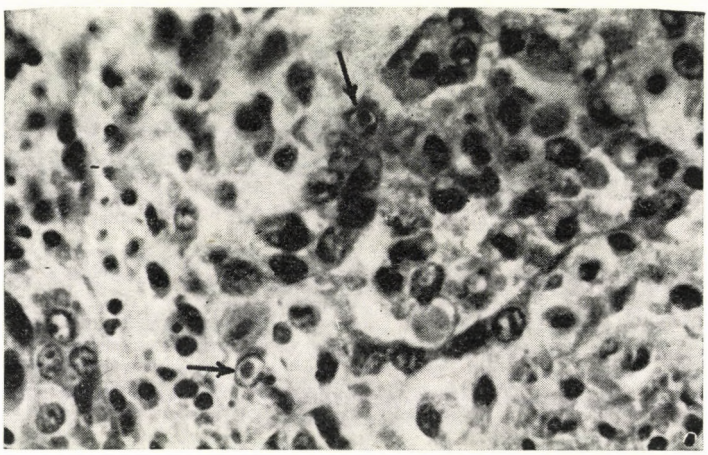

Fig. 10. Chronic duck plague. Type-A intranuclear inclusions of Cowdry in hepatocytes and RHS cells, ×800; H. and E.

Discussion

Aquatic birds (mallards, domestic ducks and geese) spontaneously died or killed in different stages of natural or experimental duck plague often showed, in addition to enteric changes, grossly visible liver lesions indicative of dystrophy during the acute stage. Ultrastructural studies revealed predominance of viral hepatocellular damages in acute cases of the disease. The hepatocellular changes appeared initially sporadically in different intralobular regions,

later involved minor groups of cells or major areas of the lobule. The main hepatocellular change was the so-called coagulation necrosis (development of dark, highly electron-dense hepatocytes — acidophil necrosis); this change was not usually associated with the appearance of intranuclear inclusions, but at a later stage a few herpesvirus particles were as a rule seen inside the highly electron-dense, damaged and mostly shrunken nucleus. Since the hepatocytes showing the ultrastructural features of coagulation necrosis occasionally enclosed the causative agent of duck plague, the conclusion lies close at hand that they represented direct target cells of the duck-plague virus. Presumably, however, the cells directly affected by the virus die in the early stage of penetration, and can, therefore, only exceptionally furnish the conditions required for virus synthesis.

The birds acutely diseased in duck plague did, however, frequently show an extensive hepatocellular degeneration involving appearance of intranuclear inclusions. The enlarged nuclei of these cells showed a characteristic rearrangement of the chromatin substance and enclosed as a rule many virus particles. During intranuclear virus synthesis, remarkable paucity of intracellular glycogen and vesicle-like dilatation of the endoplasmic reticulum was noted. Hypertrophy of the latter, and myeline degeneration of the cytoplasmic membrane were not observed in host cells invaded by the duck-plague virus. Presence of the virus within the intranuclear inclusions presented unequivocal evidence of the direct responsibility of the virus for the observed type of hepatocellular changes.

Further to the above hepatocellular lesions, hepatocytes of low electron density, consisting frequently only of nuclear and cytoplasmic residues, were also found in intralobular sites. The ultrastructural appearance of these cells indicated disintegration after the completion of virus synthesis or, probably, death by cytolysis devoid of virus synthesis. The frequent presence of considerable amounts of cell debris inside hepatolobular sinusoids and lumina of blood vessels was consequent upon hepatocellular viral damage. Indications of virus synthesis, and viral mural damage were infrequently observed also in sinusoidal endothelia. These changes, and the vascular troubles associated with viraemia, may have been responsible for the necrotic or haemorrhagic involvement of major intralobular regions, or several lobules. Vascular wall damage, and consequent haemorrhages may have been promoted, at least partly, by the considerable degree of hepatocellular degeneration.

According to our own experience, the prolonged form of duck plague observed by us is developed above all by growing and adult aquatic birds, in the form of a subacute or chronic interstitial hepatitis. During the chronic course, mesenchymal damages become predominant over hepatocellular necrosis, which appears only sporadically in the chronic stage. Part of the hepatocytes, however, show ultrastructural organelle changes (of mitochondria

and endoplasmic reticulum) and alterations of the membrane structure along the luminal sides, which account for hepatocellular functional insufficiency in respect of the processing and secretion of bilirubin. A sequel to that change is the icteric condition frequently observed in aquatic birds infected by the herpesvirus of duck plague.

The electron microscopic detection of virus particles inside the hepatocytes of birds suffering from the chronic form of duck plague is rarely successful. However, light microscopic examination of major hepatolobular areas shows the presence of intranuclear inclusions, which are of diagnostic value, in hepatocytes adjoining interlobular septa, and, less often, in proliferating RHS cells.

The morphological study of the organs of aquatic birds diseased in duck plague has shown that, against the prevailing concepts, the acute form of the disease is associated not only with circumscribed focal and haemorrhagic liver changes, but also with the development of an acute liver dystrophy directly elicited by the herpesvirus responsible for duck plague. The viral liver dystrophy is as a rule serious enough to represent the primary cause of death. Since, according to our experience, liver involvement is frequently associated with duck plague, we recommend that, further to the clinical diagnosis, electron microscopic detection of the virus in and its isolation from the liver should be attempted in each case. Isolation from the digestive tract is greatly hampered by secondary bacterial infections which are usually present.

Consideration should also be given to the fact that the liver changes developed by aquatic birds suffering from acute duck plague may occasionally become complicated by inflammatory involvement and progress thereby to a chronic hepatitis. The diagnostic identification of the chronic forms of duck plague is of great importance, on the one hand because chronic interstitial hepatitis, developed as a sequel to duck plague may occasionally be fatal in outcome, on the other hand, because part of the birds surviving chronic duck plague may become symptomless excretors shedding the virus in the flock. Transition from acute to chronic course presumably signifies a greater specific and non-specific resistance of the hosts.

It follows from the foregoing considerations that subacute or chronic hepatitis, developed by aquatic birds as a sequel to duck plague, has, like the hepatosis developed in acute cases, a decisive influence on the outcome of the primary disease.

Acknowledgements

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THE SUSCEPTIBILITY OF DOMESTIC WATERFOWLS TO NEWCASTLE DISEASE VIRUS (NDV) AND THEIR ROLE IN ITS SPREAD

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Day-old, growing and adult domestic ducks and geese were examined for susceptibility to, and excretion of, Newcastle disease virus (NDV) by intranasal and conjunctival infection with 10^6 and 10^2 ID_{50}/ml doses of the lentogenic (La Sota) and mesogenic (Hertfordshire) strains of NDV used for vaccination in Hungary, and with a velogenic NDV strain isolated from natural infection.

Examinations by virus diagnostic methods have shown that both domestic waterfowl species are susceptible to all examined strains at all ages, but to a moderate degree. Geese were more susceptible than ducks, and susceptibility tended to decrease with progressing age, whereas serological immune response, studied by the haemagglutination inhibition test, tended to increase in titre level and protective value.

The lentogenic and mesogenic vaccine strains replicated only in the pharynx, i.e. at the site of infection and in its surroundings, and were excreted exclusively in the pharyngeal discharge, for a short transitory period, at a low infective titre.

The velogenic strains gave rise to viraemia in both waterfowl species, and were excreted in all body discharges for about 8 days, but the frequency and degree of excretion decreased with progressing age. The higher dose elicited clinical disease in all day-old goslings, and killed the greater part of them after a latency of 3–8 days and a subsequent 1–5-day course of disease. In day-old ducklings and growing and adult ducks and geese, the infection remained clinically inapparent and was demonstrable only by virus-diagnostic methods.

Since only high doses of NDV were able to infect the aquatic birds, natural infection of the latter presumably requires intimate contact with massively infected gallinaceous birds. Only the velogenic NDV strains, responsible for viraemia, were excreted by diseased or massively infected aquatic birds for a longer time and in an appreciable amount in all body discharges; exactly these strains constitute a hazard to susceptible gallinaceous birds.

Long-term carriership of NDV seems to be unlikely in waterfowls.

Mediation of ND by waterfowls threatens only in small flocks or in nature, and can be minimized by separation of the feeding and rest place of the birds, and isolation of the flock during epidemic period.

Newcastle disease (ND) has been the economically most important disease of domestic poultry for half a century.

ND has been recurrent on few to many occasions wherever poultry has been bred (Lancaster, 1963, 1977; Hanson, 1978), and its control has been a matter of great concern in practically all countries. Investigations into newer aspects of ND are still being published in the literature (Alexander et al., 1979; Allan et al., 1978; Balla and Végh, 1976; Lomniczi and Derzsy, 1964; Manninger and Mészáros, 1975; Mészáros, 1976; etc.).

Despite the vast amount of information accumulated on ND, and the well-established systems for its regular control, the economic importance of ND still tends to increase, for various reasons, such as broad host range,

multiple modes of spread, and inadequate knowledge of some epizootiological aspects.

ND had originally been observed in South Asia and in England in 1926; by the 'thirties, it became known in several continents (Kranevald, 1926; Picard, 1928; Doyle, 1948). Its occasional occurrence in waterfowls has been known since the 'forties (Asplin, 1947; Blaxland, 1951; Elek and Prokopovitsch, 1948; Rend, 1947; Wilson, 1950).

Involvement of waterfowls by ND is, however, still a rarity which deserves reporting. During recent years, mainly North-American investigators have studied waterfowls for susceptibility to, and carriership of, ND (Alexander, 1980; Bradshaw and Trainer, 1966; Kingston and Dharsana, 1979; Page, 1958; Palmer and Trainer, 1970; Rosenberger et al., 1975; Spalatin and Hanson, 1975; Webster et al., 1976).

To throw more light on certain obscure epizootiological aspects, we examined domestic waterfowls for susceptibility to, and shedding of, ND, for the most part in model experiments.

Materials and methods

Our studies were based on model experiments and isolation experiments from diagnostic material. In the model experiments we used the lentogenic (La Sota) and the mesogenic (Hertfordshire) strain used as vaccine strains in Hungary, and velogenic strains isolated from natural cases (Tanyi and Sári, 1970). The velogenic isolates were purified, identified, titrated, and stored for the period of study as recommended in the literature (Mayr et al., 1977; Anon., 1971; Röhrer, 1968; WHO Expert Committee, 1971).

Each ND virus (NDV) strain was used for experimental infection in 2 dilutions, 10^6 ID_{50} for a high dose (group T) and 10^2 ID_{50} for a lower dose (group H). The infectious material was applied intranasally and on the conjunctiva.

The scheme of the model experiments is shown in Table I. Three age groups (day-old, 2 to 4 weeks old, over 6 months old) of ducks and geese were infected at the above dose levels of each virus strain, and in-contact controls were set up with each lot. Thus the model experiments numbered 9, and each covered 4 experimental groups.

All bird groups were observed for 6 weeks, by monitoring clinical state, gross lesions, pharyngeal and cloacal swabs, and serum samples.

Swabs were taken on 5 consecutive days following experimental infection, twice during the second week, and then at weekly intervals. Blood samples were withdrawn before experimental infection, and then at weekly intervals (Table II).

Table I

Scheme of the experiments

Virus strains	Dose ID_{50}/ml	Day-old		Growing		Adult	
		duck-lings	gos-lings	duck-lings	gos-lings	duck-lings	gos-lings
		Experiment 1		Experiment 2		Experiment 3	
Lentogenic	10^2 (group H)	5+2	5+2	5+2	5+2	5+2	5+2
	10^6 (group T)	5+2	5+2	5+2	5+2	5+2	5+2
		Experiment 4		Experiment 5		Experiment 6	
Mesogenic	10^2 (group H)	5+2	5+2	5+2	5+2	5+2	5+2
	10^6 (group T)	5+2	5+2	5+2	5+2	5+2	5+2
		Experiment 7		Experiment 8		Experiment 9	
Velogenic	10^2 (group H)	5+2	5+2	5+2	5+2	5+2	5+2
	10^6 (group T)	5+2	5+2	5+2	5+2	5+2	5+2

Table II

Scheme of sampling

	Days after inoculation											
	0	1	2	3	4	5	8	11	15	22	29	36
Blood	+						+	+	+	+	+	+
Swabs (pharyngeal, cloacal)		+	+	+	+	+	+	+	+	+	+	+
Organs									airways			
									brain			
									liver–spleen			
									intestinal tract			

Virus isolation was attempted from swabs and organs by inoculation into the allantoic cavity of embryonated hen's eggs. Isolation experiments from 4 organs followed upon spontaneous death or killing at the conclusion (at 36 days) of the experiment. Embryos died in the egg, or recovered from eggs opened 96 h after infection, were examined for gross lesions, and the chorioallantoic fluids were tested for haemagglutination.

The serum samples were examined mainly by haemagglutination inhibition (HI) test.

In the case of day-old goslings, further examinations were required to obtain precise information on susceptibility to NDV. For this purpose, additional experimental groups were set up for treatment with serial dilutions of

the velogenic strain used in the main experiment, and of 10 other velogenic strains.

Studies of natural cases covered organ and blood samples of ducks and geese, submitted to this Institute for diagnostic examination between January 1979 and 30 May 1981. In these cases we used the same virus isolation and serological techniques as in the model experiments.

Results

Clinical symptoms and gross lesions

The lentogenic and mesogenic strains did not give rise to clinical illness in any age group of ducks and geese at either dose level.

All day-old goslings infected with the higher dose (10^6 ID_{50}/ml) of the velogenic strain and one in-contact bird (group T) developed clinical ND, and 5 of them died after a 1 to 3-day course of illness between days 4 and 7 after infection. Day-old ducklings infected with the same dose remained healthy, and neither ducklings nor goslings showed clinical response to the lower dose (group H). Part of the growing geese treated with the higher dose of the velogenic strain developed transitory listlessness and inappetence, but all recovered. All other growing and adult birds were healthy throughout the period of study.

The day-old goslings which developed clinical disease in response to infection with the velogenic strain showed, apart from serious general symptoms, dyspnoea, diarrhoea, weakness to the degree of immobility, and ascending paresis. At post-mortem examination empty digestive tract, mild to severe gastroenteritis, nasal catarrh, pulmonary oedema and hyperaemia, and, in a few cases here and there cardiac haemorrhages were found.

Virus isolation

In the groups treated with the higher dose of the lentogenic or mesogenic strains (groups T), the virus was isolated exclusively from the pharyngeal swab of 1–3 ducks or geese of the different groups on 1–3 occasions, between 24 and 72 h after infection. Isolation failed throughout in birds inoculated with the lower dose (groups H) of lentogenic and mesogenic NDV and their contacts, in all age groups.

The virus was re-isolated from both pharyngeal and cloacal swabs of all day-old goslings treated with the higher dose of velogenic virus and their contacts (groups T), with the exception of a single bird. In the duckling group so treated, the virus could be re-isolated only from 2 birds, 3 and 4 days

Table III

Virus isolation from pharyngeal and cloacal swabs of day-old ducklings and goslings infected with 10^6 ID_{50}/ml of the velogenic strain

Designation of birds	Days after infection																							
	1		2		3		4		5		8		11		15		22		29		36			
	Ph	C	Ph	C	Ph	C	Ph	C	Ph	C	Ph	C	Ph	C	Ph	C	Ph	C	Ph	C	A	B	L	I
Tdu 1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Tdu 2	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Tdu 3	—	—	—	—	+	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Tdu 4	—	—	—	—	+	—	+	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Tdu 5	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Tdu 6	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Tdu 7	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Tgo 1	—	—	—	+	—	+	+	—													+	+	+	—
Tgo 2	—	—	—	—	—	+	+	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Tgo 3	+	—	+	—	—	+	+	+													+	+	+	+
Tgo 4	—	—	+	—	+	+	+	+	+	+											+	+	+	+
Tgo 5	+	—	—	—	+	+	+	+													+	+	+	+
Tgo 6	—	—	—	—	—	+	+	+	+	+											+	+	+	+
Tgo 7	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—

Ph = Pharyngeal swabs; C = cloacal swabs; A = airways; B = brain, L = liver + spleen; I = intestinal tract

after infection, respectively. The birds which succumbed to the infection had excreted the virus for at least 2 days (Table III). The virus was also re-isolated from all organ samples, with the exception of the intestines of one bird. The results of re-isolation in chicken embryos are shown in Table IV.

In the day-old groups treated with the lower dose of the velogenic virus (groups H) isolation was successful only from a single gosling, between 3 and 5 days after infection.

Table IV

Isolation of NDV in embryonated hen's eggs from the organs of day-old goslings died in infection with 10^6 ID_{50}/ml velogenic NDV

	Organs									
	Airways							Brain		
	+	+	+	+	+	+	+	+	+	FID
Tgo 1	40	40	40	40	40	48	48	72	72	
	+	+	+	+	+	+	+	FID	FID	FID
Tgo 3	40	40	40	40	40	52	52			
	+	+	+	+	+	+	+	+	—	—
Tgo 4	48	48	48	48	48	48	48	48		
	+	+	+	+	+	+	+	—	—	—
Tgo 5	40	40	40	40	40	72	72			
	+	+	+	+	+	+	+	+	+	+
Tgo 6	48	48	48	72	72	48	48	48	72	72

FID = fatal intercurrent disease of the embryo, preventing evaluation

	Liver–spleen						Intestinal tract			
	+	+	FID	FID	FID	FID	—	—	—	—
Tgo 1	48	48								
	+	+	+	+	+	+	FID	—	—	—
Tgo 3	40	40	48	48	48	72				
	+	+	+	+	FID	+	+	+	—	—
Tgo 4	48	48	72	72		24	48	48		
	+	+	+	+	+	+	FID	—	—	—
Tgo 5	48	48	48	48	72	48				
	+	—	—	—	—	+	FID	—	—	—
Tgo 6	72					72				

Table V

Isolation of NDV from pharyngeal and cloacal swabs of growing ducks and geese infected with 10^6 ID_{50}/ml of velogenic virus

Designation of birds	Days after infection																							
	1		2		3		4		5		8		11		15		22		29		36			
	Ph	C	Ph	C	Ph	C	Ph	C	Ph	C	Ph	C	Ph	C	Ph	C	Ph	C	Ph	C	A	B	L	I
Tdu 1	—	—	+	—	+	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Tdu 2	—	—	—	—	—	+	—	+	—	+	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Tdu 3	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Tdu 4	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Tdu 5	+	—	+	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Tdu 6	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Tdu 7	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Tgo 1	—	—	—	—	+	+	+	—	—	—	—	—	—	—	—	—					—	—	—	—
Tgo 2	—	—	—	—	—	+	+	+	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Tgo 3	—	—	+	+	+	+	+	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Tgo 4	+	—	+	—	—	—	—	—	—	+	—	—									—	—	—	—
Tgo 5	—	—	—	+	+	+	—	—	FID	FID	FID	—	—	—	—	—	—	—	—	—	—	—	—	—
Tgo 6	FID	FID	—	—	—	—	—	—	—	FID	—	—	—	—							—	—	—	—
Tgo 7	—	—	—	—	—	—	—	—	FID	FID	—	—	—	—	—	—	—	—	—	—	—	—	—	—

FID = fatal intercurrent disease

The isolation was positive between days 1 and 5 after infection from swabs of all 5 growing geese inoculated with the higher dose. The second re--isolation followed 24 h after the first one in one case, but usually after a 2-day negative interval upon isolations between 48 and 96 h post-inoculation.

The virus was alternately present in the pharyngeal and cloacal swabs, but the percentage of positive samples was approximately identical in the two sample categories. No virus could be isolated from the 2 in-contact birds allocated to that group of growing geese.

In group T of growing ducks re-isolation was successful from 3 birds during the first 5 days; in 2 cases only from the pharyngeal swabs, and in 1 case only from the cloacal swab (Table V).

Isolation failed from all growing ducks and geese of groups H except from a single goose, which yielded positive cloacal swabs on days 4 and 5 after inoculation.

In the adult age group, one experimental and one in-contact goose of group T yielded a positive pharyngeal swab 48 and 96 h after inoculation. The isolation experiments had negative results in all other instances.

Table VI

HI antibody titres in the sera of day-old ducklings and goslings infected with 10^6 ID_{50}/ml velogenic virus

Designation of birds	Days of sampling p.i.						
	0	8	11	15	22	29	36
Tdu 1	0	0	0	0	0	0	0
Tdu 2	0	0	0	0	0	0	0
Tdu 3	0	0	0	0	0	0	0
Tdu 4	0	4	2	2	0	0	0
Tdu 5	0	2	0	0	0	0	0
Tdu 6	0	0	0	0	0	0	0
Tdu 7	0	0	0	0	0	0	0
Tgo 1	0						
Tgo 2	0	32	16	16	8	8	—
Tgo 3	0						
Tgo 4	0						
Tgo 5	0						
Tgo 6	0	0					
Tgo 7	0	128	64	64	32	32	16

Remark: The tests were carried out with 4 HA units of the La Sota and the homologous strain. The titres are expressed as reciprocals of the highest positive serum dilution

Serological tests

The HI titres of the geese increased over those of the ducks in the groups treated with 10^6 ID_{50}/ml virus (groups T). The titres of the survivors were relatively low in the day-old gosling group, higher in the growing goose group, and occasionally very high in the adult geese. The antibody titres tended to increase with progressing age in both geese and ducks, but the species-specific difference persisted throughout. Transmission of the infection by contact occurred frequently among geese, but failed to occur among ducks. The HI titres reached a peak soon, between 8 and 11 days, then dropped abruptly (Tables VI–VIII).

Table VII

HI antibody titres in the sera of growing ducks and geese infected with 10^6 ID_{50}/ml velogenic NDV

Designation of birds	Days of sampling p.i.						
	0	8	11	15	22	29	36
Tdu 1	0	8	16	4	8	4	2
Tdu 2	0	2	8	16	16	4	2
Tdu 3	0	2	0	8	8	4	2
Tdu 4	0	0	0	0	0	0	2
Tdu 5	0	16	16	8	4	0	4
Tdu 6	0	0	0	0	0	0	0
Tdu 7	0	0	0	0	0	0	0
Tgo 1	0	256	32	32			
Tgo 2	0	64	32	16	8	8	4
Tgo 3	0	32	32	16	8	8	4
Tgo 4	0	64					
Tgo 5	0	32	128	32	16	8	4
Tgo 6	0	32	128				
Tgo 7	0	0	64	8	16	4	4

See Remark to Table VI

Only a few birds showed HI titres of a relatively low level in the groups treated with the lower dose (groups H).

Infection experiments with velogenic strains

Infection experiments in day-old goslings with serial dilutions of the velogenic strain used in the main experiments

Mortality tended to increase, and the latency and course of the disease tended to decrease as the virus dose was risen (Table IX).

Table VIII

HI antibody titres in the sera of adult ducks and geese infected with 10^6 ID_{50}/ml velogenic NDV

Designation of birds	Days of sampling p.i.						
	0	8	11	15	22	29	36
Tdu 1	0	64	128	64	16	8	4
Tdu 2	0	32	128	64	16	8	4
Tdu 3	0	128	512	256	32	16	4
Tdu 4	4	16	16	16	8	8	8
Tdu 5	0	32	32	16	8	4	4
Tdu 6	0	0	0	0	0	0	0
Tdu 7	0	0	0	0	0	0	0
Tgo 1	0	128	256	128	128	64	32
Tgo 2	0	128	256	64	128	32	32
Tgo 3	0	1024	1024	512	512	128	32
Tgo 4	0	1024	1024	128	256	128	16
Tgo 5	0	64	128	128	32	32	8
Tgo 6	0	256	256	256	128	64	32
Tgo 7	0	128	128	512	512	32	32

See Remark to Table VI

Table IX

Results of experimental infection of day-old goslings with serial dilutions of velogenic NDV

Dose ID_{50}/ml	Died/infected birds	Diseased/healthy birds	Mean latency time in h/n
10^7	8/10	8+1/1	84/8
10^6	8/10	8+1/1	96/8
10^5	5/10	5+3/2	128/5
10^4	4/10	4+2/4	163/4
10^3	2/10	2+1/7	—
10^2	0/10	1/9	—

The latency period lasted 3–8 days, and most deaths occurred between 24 and 48 h after the first appearance of symptoms. Some birds of those which had been ill for 3 days recovered.

The symptoms and gross lesions were essentially the same as in the main experiments.

Infection experiments with different velogenic strains

Groups of 10 goslings, serologically negative in the HI test, were infected with the velogenic strains I–X. The greater part of the birds (7 to 10 in 10) died 3–8 days after inoculation. In 8 groups, 1 to 3 birds showed signs of recovery or were free from symptoms throughout.

The strain used for experimental infection could be re-isolated from all carcases.

Diagnostic studies in natural cases of ND

The organ and blood samples of nearly all naturally diseased ducks and geese submitted to the Department of Virus Diagnostics, Veterinary Institute of Debrecen, between January 1979 and 30 May 1981, were examined for NDV. These studies covered organ samples of a total of 3779 ducks and geese originating from 250 large or small flocks, and blood samples of a total of 657 ducks and geese from 69 large or small flocks. The flocks were located for the most part in counties Szabolcs-Szatmár, Hajdú-Bihar and Szolnok; a minor part of the samples originated from other regions of Hungary. The flocks of origin belonged to different age groups. All organ samples were derived from birds died of diseases other than ND. The blood samples were collected from healthy birds.

No NDV could be isolated from any organ or blood sample.

Discussion

All three age groups of ducks and geese proved to be susceptible to infection by NDV. However, large doses of virus were required to produce an infection. Susceptibility also varied between strains, host species, and age groups.

Our experimental observations indicate that the vaccine strains replicated probably only at the sites of infection, and were excreted in the nasal and pharyngeal discharges for a short transitory period, for 72 h at most, at a low infective titre, and were further on no longer demonstrable in the organism. Their antigenicity seemed to be low when checked in natural infections. Enzootic occurrence of lentogenic and mesogenic strains seems unlikely also within a given waterfowl population.

Against this, the velogenic strain gave rise in day-old birds to viraemia, which elicited clinical disease after 3–8 days of latency, depending on the virus titre, and the diseased birds shed the virus at relatively high infective titres, in all body discharges. The growing birds developed a transitory illness, whereas the adult birds only an inapparent infection, in response to the velogenic strain. Thus, susceptibility tended to decrease while the immune response tended to increase, with progressing age.

Geese proved to be more susceptible to NDV than the ducks, since in the day-old age they had been rendered ill with great certainty by the higher dose used, to which the day-old ducks did not respond by clinical symptoms. During the growing age, dissimilar susceptibility was indicated by the greater number of virus isolations and higher serum antibody titres among the geese.

The susceptibility of waterfowls to velogenic NDV strains has been studied by other investigators in both natural and experimental infections (Friend and Trainer, 1972; Raszevszka, 1966; Weidenmüller, 1972; and others). Circulation of lentogenic strains, possessing a thermostable haemagglutinin, among wild geese indigenous at the Atlantic Coast has been reported by Rosenberger et al. (1975), and Spalatin and Hanson (1975). Our own investigations have substantiated these findings, and also disclosed some new information on the behaviour of mesogenic strains and lentogenic strains possessing thermolabile haemagglutinin.

Since domestic ducks and geese proved to be susceptible to NDV strains, independent of virulence of these, at all ages, and evidence has been presented of shedding of these strains by them, they may well serve as reservoirs and transmitters of NDV infection in poultry flocks.

However, the epizootiological importance of waterfowls in the spread of ND varies with the species and age of the host, and with the degree of virulence of the NDV strain involved as well.

We agree with other authors (Friend and Trainer, 1972; Lancaster, 1963, 1977) that large doses of NDV are required to produce an infection in aquatic birds, and such a massive infection could occur in natural conditions only by intimate contact with latently infected or clinically diseased hosts.

The degree of virus excretion was closely related to the species-specific susceptibility (goose) and age (day-old, growing) of the waterfowl host, and also depended on the degree of virulence of the NDV strain used.

A long-term NDV carriership of domestic ducks and geese seems unlikely if the low susceptibility of these species on the one hand, and the speedy development of high antibody titres on the other are taken into consideration.

The epizootiological, clinical, and pathological–anatomical features of ND seemed to be non-characteristic in ducks and geese, thus virus isolation remains the only reliable diagnostic approach.

Diagnostic evidence of the responsibility of NDV for outbreaks in small poultry flocks and among wild birds requires virus isolation from, and serological screening of, ducks and geese, and occasionally, the use of day-old goslings as test birds for contact infection.

During seasonal outbreaks, separation of the feeding and resting places of small flocks and temporary isolation of the flock may prevent the transfer of NDV infection by domestic waterfowl.

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COMPARATIVE STUDIES ON HAEMAGGLUTINATING ACTIVITY AND IMMUNOGENICITY OF BOVINE PARAINFLUENZA-3 VIRUS STRAINS

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Studies on parainfluenza-3 (PI-3) virus strains isolated from 6–8 weeks old and 5–12 months old calves affected by different respiratory diseases revealed that isolates of identical infective titres markedly differed in their haemagglutinating (HA) activity, and that virus strains maintained this property through several passages.

PI-3 virus strains of high HA activity were exposed to physical or chemical treatments. Heat treatment at 50 °C for 120 min or treatment with 0.5% trypsin for 30 min destroyed both the infectivity and the HA activity of the virus. Other treatments, among others, heat treatment at 50 °C for 80 min or treatment with 0.25% trypsin for 30 min, destroyed the HA activity of the virus but did not affect its infectivity. Formaldehyde as well as beta-propiolactone treatment destroyed the infectivity of the virus and damaged its haemagglutinins to different extents. Ethylenimine (EI) was found to inactivate the infectivity of PI-3 virus but failed to affect its HA activity.

The immunogenicity of virus strains of originally high and originally low HA activity and damaged in different ways showed a positive correlation with the HA activity of the strains.

Rabbits inoculated twice with an EI-inactivated PI-3 virus developed haemagglutination-inhibiting (HI) antibodies already after the first vaccination. However, marked elevation of virus-neutralizing (VN) antibody titres needed a second vaccination.

During investigations on acute respiratory diseases of contagious nature in calves, parainfluenza-3 (PI-3) virus strains were isolated from different disease entities. Several biological characteristics of the virus strains were comparatively studied.

Materials and methods

Virus strains. PI-3 virus strains were isolated from several weeks old calves with respiratory disease (Köves et al., in press), and from adult bulls affected by acute respiratory disease (epizootic coughing) (Bartha and Köves, 1975). Some data for the PI-3 virus isolates are given in Table I. The isolated strains were purified by the endpoint-dilution method performed three times. Infective titres were determined in secondary calf kidney cell cultures.

Haemagglutinating (HA) activity. Virus-containing maintenance medium (Earle's solution, free of serum, Difco) of cell cultures was centrifuged at 4000 rpm for 30 min (Janetzki K23 centrifuge). The HA titre of isolate samples prepared in this way was examined both in tubes and in microtitrator (Ta-

kátsy) trays at 4, 22 and 37 °C. In the main experiments, tube agglutination was used; the incubation period was 60 min at 37 °C and 240 min at 22 °C. In the tests 0.5% suspensions of washed cattle and guinea pig erythrocytes were used.

Physicochemical treatments. Cell-free supernatants of virus strains propagated in cell cultures were exposed to UV irradiation for 10 and 20 min (BLF-32 bactericidal lamp, LABOR MIM Budapest). Exposure to freon gas was performed in LABOR MIM GST-21 gas sterilizers. Virus strains were treated with 0.2% sodium dodecyl sulphate (SDS) for 60 and 120 min, with 1 M $MgCl_2$ for 10 and 20 min at 50 °C, with 0.25% trypsin solution (Difco) for 30 and 60 min, and with 0.5% trypsin solution for 60 min. Samples were heat-treated at 50 °C for 80, 100 and 120 min. Formaldehyde, beta-propiolactone and ethylenimine (EI) treatments were performed at 37 °C, with concentrations of 400 μg/ml, 1000 μg/ml and 400 μg/ml, respectively. For the immunization experiments, each virus suspension was mixed with an equal volume of the incomplete oil adjuvant (HUMAN Institute for Serobacteriological Production and Research, Budapest). Five rabbits, each weighing 2.5–3.0 kg, were given two 1 ml doses of one of the preparations intramuscularly, at an interval of two weeks. Serum antibody titres of rabbits were determined by virus neutralization (VN) and haemagglutination-inhibition (HI) tests.

Table I

Haemagglutinating (HA) activity of PI-3 virus strains

Strain	Calf		Infective titre 10^x/0.1 ml	Haemagglutination*	
	Age	Disease entity		cattle erythrocytes	guinea pig erythrocytes
4/5	12 months	E.C.	6.5	1:32	1:256
9/4	5–7 ,,	a.E.C.	6.5	32	256
2/4	2–3 ,,	a.E.C.	6.0	16	128
1/4	2–3 ,,	a.E.C.	6.5	2	4
12/4	5–7 ,,	a.E.C.	6.5	32	128
ME 2/4	2–4 ,,	G.R.D.	6.0	4	8
ME 6/5	2–4 ,,	G.R.D.	6.5	32	128
ME 9/6	2–4 ,,	G.R.D.	6.5	16	64
ME 9/7	2–4 ,,	G.R.D.	6.5	32	256
ME 6/6	2–4 ,,	G.R.D.	6.0	32	128

* tube agglutination. The incubation period was 60 min at 37 °C, and 240 min at 22 °C
a.E.C. = status after epizootic coughing
E.C. = epizootic coughing
G.R.D. = general respiratory disease

Results

The infective and HA titres of the PI-3 virus isolates were studied during six passages in cell culture. It was found that throughout the passages the HA titre of strains having an identical infective titre did not show alterations, as compared to each other. The infective and the HA titres of virus strains in the fourth passage are shown in Table I.

The two strains of high HA activity were exposed to different physical and chemical agents. Strains 4/5 and ME 9/7 behaved uniformly in these experiments; results are summarized in Table II. Immune responses induced

Table II

Infective titre and haemagglutinating activity of strain 4/5 after physical and chemical treatments

Treatment	Infective titre 10^x/0.1 ml	Haemagglutination	
		cattle erythrocytes	guinea pig erythrocytes
Untreated 4/5 strain	7.0	1:64	1:512
UV 10 min	—	64	64
UV 20 min	—	8	16
Freon gas 60 min	—	64	128
Sodium dodecyl sulphate, 0.2%, 60 min	3.0	32	64
Sodium dodecyl sulphate, 0.2%, 120 min	3.0	16	64
1 M $MgCl_2$ 50 °C, 10 min	2.0	64	256
1 M $MgCl_2$ 50 °C, 20 min	—	32	64
Trypsin 0.25%, 30 min	2.0	—	—
Trypsin 0.25%, 60 min	1.0	—	—
Trypsin 0.5%, 30 min	—	—	—
50 °C, 80 min	3.5	—	—
50 °C, 100 min	2.0	—	—
50 °C, 120 min	—	—	—
HCHO 400 μg/ml 20 h	—	32	128
BPL 1000 μg/ml 20 h	—	32	128
EI 400 μg/ml 20 h	—	64	512

in rabbits by strains of originally high, originally low (untreated) and variously impaired (treated) HA activity were studied. The immunogenicity of untreated live virus suspensions and of virus suspensions subjected to different treatments

Table III

Antigenicity studies in rabbits. I

Inoculum	Antibody titre			
	Day 0	Day 14	Day 28	
	VN	VN	VN	HI
1/4 live	—	—	18.6	8
4/5 live	—	—	122.3	256
4/5 EI	—	—	128	248.3
4/5 freon	—	—	28	56.8
4/5 UV 10 min	—	—	64	64
4/5 50 °C 120 min	—	—	—	—
4/5 0.5% trypsin	—	—	—	—
4/5 HCHO	—	—	22.6	21.3
4/5 BPL	—	—	32	21.3

was examined by VN tests performed on days 0, 14 and 28, and by a HI test performed on day 28 (Table III).

The immunogenic potential of the live virus strain 4/5 and that of the virus treated with the conventional inactivating agents or with EI (which is the least active in damaging the HA antigen) was compared after repeated inoculation experiments in rabbits. Serum VN titres of rabbits were determined on days 0, 14 and 42, and HI titres on day 42 (Table IV).

Table IV

Antigenicity studies in rabbits. II

Inoculum	Antibody titre			
	Day 0 VN	Day 14 VN	Day 42	
			VN	HI
4/5 live	—	—	85.3	277.3
4/5 EI	—	—	74.6	206.6
4/5 BPL	—	—	13.3	37.3
4/5 HCHO	—	—	10.6	21.3

The development of immune response induced by the EI-inactivated strain was studied after immunizing experimental rabbits twice, at an interval of 14 days. The courses of the VN and HI antibody responses are shown in Fig. 1.

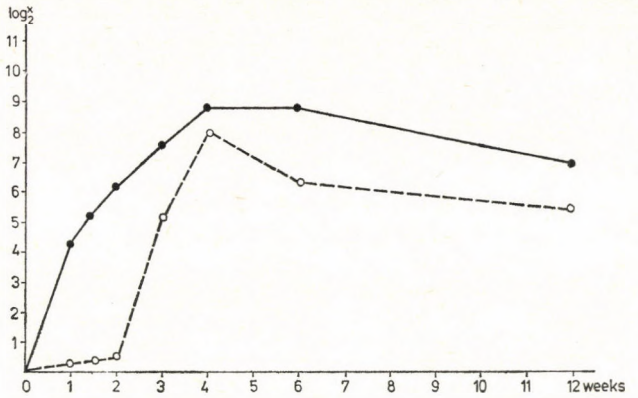

Fig. 1. VN and HI titres of rabbits vaccinated twice at an interval of 14 days. Solid line, HI titre, broken line, VN titre

Discussion

The PI-3 virus isolates of identical cell culture passage number and identical infective titre considerably differed from one another in HA activity. The differences were more pronounced if guinea-pig erythrocytes than if cattle erythrocytes were used in the HA test. The HA titre of the virus strains remained constant and characteristic of the strains during further passages. No relationships were found between this property of the viruses and the different epizootiological situations of which the isolates had been obtained.

Scheid et al. (1972) and Scheid and Choppin (1973) established that a single glycoprotein ("HN") was responsible for the neuraminidase (NA) and HA activities of paramyxoviruses. Later on, it was revealed that peplomers with a HN activity primarily had a role in adsorption of PI viruses to cells, while another glycoprotein, protein F, was responsible for their penetration (Merz et al., 1980; Scheid et al., 1978). In our experiments, we damaged to different degrees the HA antigen of PI-3 virus strains of high HA activity. Some drastic treatments destroyed both the infectivity and the HA activity. Other treatments diminished the HA activity of viruses sooner than their infectivity. In these cases, viruses completely lost their detectable HA activity, but partly retained their infectivity (although the latter also decreased) (Table II). Despite marked or apparently complete inactivation of HA activity of the HN glycoprotein, some virions may be presumably able to penetrate the cells and replicate there. Formaldehyde and beta-propiolactone treatment, on the other hand, destroyed virus infectivity and damaged HN antigens to different degrees. This finding is partly consistent with the observations of Morein and Bergmann (1977), who found an increase in NA activity together with a decrease in HA activity after formaldehyde treatment. However, in

their experiments beta-propiolactone failed to influence the HA activity of the virus. It is remarkable that in our experiments EI destroyed virus infectivity but failed to affect HA activity.

Morein et al. (1973) studied the immunogenicity of virus strains of high and low NA activity, and found that the antibody response detectable in the serum and the nasal secretion was independent of the NA activity of strains. They observed that, together with the increase of HI titres, anti-NA titres also increased, irrespective of the degree of the NA activity of the strains. In our studies, the immunogenicity of PI-3 virus strains of originally high and originally low HA activity showed a positive correlation with the HA activity of the strains. Thus, strain 4/5 of high HA activity induced a far more expressed immune response than strain 1/4, which had a low HA activity. Immunogenicity of the treated virus preparations also showed a positive correlation with the degree to which their HA activity was damaged (Tables III and IV).

By immunizing rabbits twice with the EI-inactivated strain of high HA activity (EI being the agent which affects the surface antigens to the least extent), an expressed immune response was induced.

In the HI test, marked antibody responses were observed already after the first vaccination and the titres increased further after the second vaccination. In the VN test no, or very slight, antibody responses were demonstrated after the first vaccination. However, the second vaccination was followed by a striking increase in the VN antibody titres (Fig. 1).

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IMMUNIZATION EXPERIMENTS WITH AN INACTIVATED PARAINFLUENZA-3 VIRUS

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Characteristics of the immune response induced by an ethylenimine-inactivated PI-3 virus strain of high HA activity were studied. The strains had been isolated from calves showing symptoms of respiratory disease. Calves were vaccinated twice at an interval of 7, 10, 14 or 21 days. The curves representing the HI (haemagglutination-inhibiting) and VN (virus-neutralizing) antibody responses did not run parallel. The HI antibody titres increased significantly in all programmes, whereas VN antibodies appeared only after the second vaccination and their titre reached the highest level in the programme in which the interval between the two injections was 14 days. On the 14th and 28th days after re-vaccination it was found that re-vaccination performed after an interval of 14 days was significantly more effective than that carried out after an interval of 7, 10 or 21 days, as regards VN antibodies. The HI antibody response showed no marked differences between the programmes.

Antibody responses following immunization of lambs with PI-3 virus were also studied. When antibody titres started to decrease, the lambs were challenged with PI-3 virus. Unvaccinated animals became affected by the challenge dose, were shedding the virus and showed histopathological changes. The immunized animals, though showing minor histopathological changes, did not exhibit clinical symptoms and did not shed the virus. Marked proliferation of cells responsible for cell-mediated immunity was observed in the lymphoid organs.

The results indicate that vaccination with the ethylenimine-inactivated PI-3 virus of high HA activity can provide protection against PI-3 virus infection.

In the preceding paper (p. 45 in this volume), striking differences found in the haemagglutinating (HA) activity of bovine parainfluenza-3 (PI-3) virus isolates have been reported. A correlation was found between the HA activity and the immunogenicity of the strains.

Characteristics of the immune response induced by an ethylenimine-inactivated preparation of a virus strain of high HA activity are reported in the present paper.

Materials and methods

Vaccine. The PI-3 virus strain 4/5 of high HA activity was isolated from calves affected by acute respiratory disease and it was propagated in calf kidney (CK) cell cultures. The virus was inactivated with ethylenimine and oil-adjuvated (see p. 45 in this volume).

Immunization of calves. Four groups of 6 to 8 weeks old Holstein–Friesian bull calves of identical origin were used in the experiments. Each group consisted of 5 calves. Each calf was given two 5 ml doses of the inactivated vaccine intramuscularly. The time interval between the two injections

was 7, 10, 14 and 21 days, respectively, in the four groups. The immune response was assessed by haemagglutination-inhibition (HI) and virus-neutralization (VN) tests.

The significance of antibody level differences between the groups was determined by Student's *t* test.

Immunization and challenge infection of lambs. Eight-week-old Merino lambs were immunized twice with 2 ml of the vaccine, at an interval of 10 days. On day 105 after the first vaccination, 3 immunized and 3 untreated lambs were challenged intranasally and intratracheally, each with twice 2 ml (2×10^8 $TCID_{50}$) of the virus strain 4/5. The challenge infection was repeated 24 h later. Three lambs were used as uninfected controls.

Re-isolation of virus was attempted from nasal swabs taken daily, and from samples taken from the mucous membrane of nasal conchae, from the trachea, from the lungs and from pectoral lymph-nodes of lambs bled on days 7 and 8 postinfection, in secondary calf kidney cell cultures.

Histopathological examinations. Five-μm-thick sections were prepared from the organs fixed in 10% formaldehyde and embedded in paraffin. The sections were stained with haematoxylin and eosin.

Results

In the 5th passage, before inactivation, the PI-3 virus strain 4/5 had an infective titre of 10^7 $TCID_{50}$/0.2 ml and agglutinated guinea-pig erythrocytes up to the dilution 1:1024. Inactivation failed to alter the HA activity of the virus.

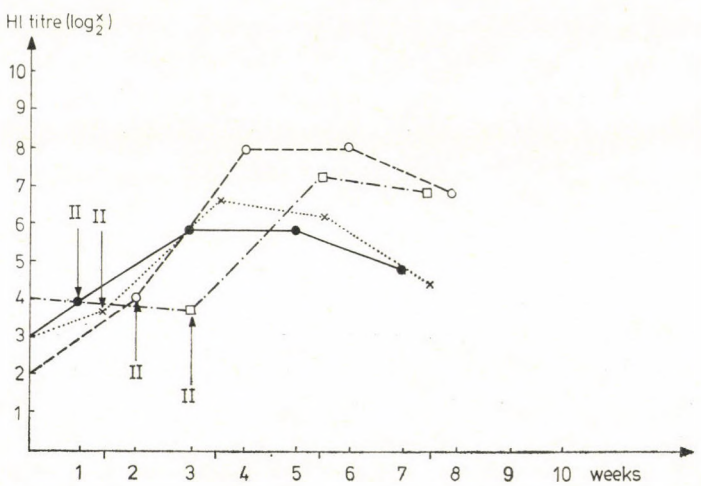

Fig. 1. Haemagglutination inhibition in calves immunized at intervals of 7, 10, 14 or 21 days (II: time of second vaccination)

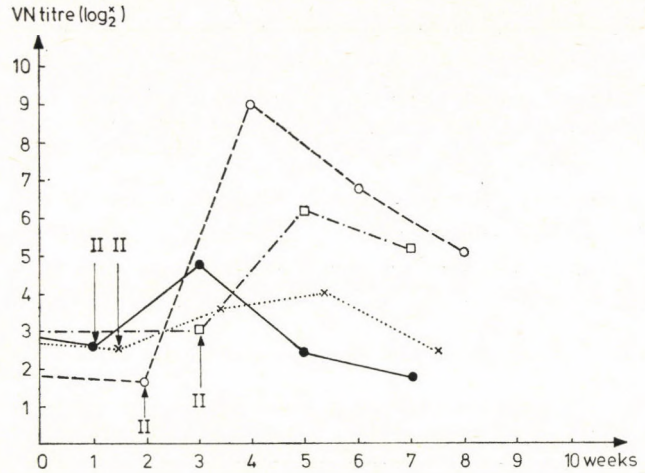

Fig. 2. Virus neutralization in calves immunized at intervals of 7, 10, 14 or 21 days (II: time of second vaccination)

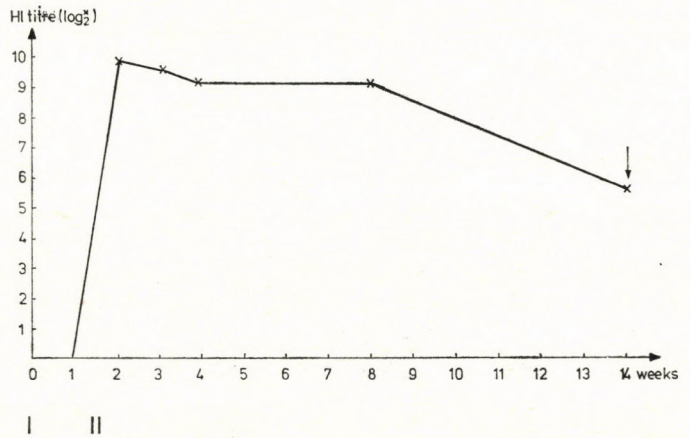

Fig. 3. Haemagglutination inhibition in immunized lambs (I: first vaccination, II: second vaccination, arrow: challenge infection)

HI antibody titres found in sera of calves given two injections of inactivated virus at different intervals are shown in Fig. 1 and VN antibody titres measured at the same time in Fig. 2. In evaluating the postvaccination humoral immune responses (HI and VN antibodies), the antibody titres appearing after injections repeated at different times were determined.

Antibody titres were determined at the time of the second vaccination, and subsequently on every 14th day, and were expressed as mean power indices of $\log_2$. The significance of antibody titre elevation is summarized in Table I. The comparative significance of the different programmes, as related to one another, was evaluated separately for HI and VN antibodies (Table II).

HI antibody titres of the 3 immunized lambs used in the challenge experiments are indicated in Fig. 3. The unvaccinated lambs showed a febrile response (40.1–40.7 °C) on days 3 and 4 postinfection. One lamb showed clinical symptoms including a serous nasal discharge, sneezing and dullness from the 5th day postinfection. The condition further aggravated by the 6th day when the lamb exhibited severe coughing and dyspnoea. The other two lambs also showed dullness, nasal discharge, sneezing and coughing. On day 7, the severely affected lamb was bled. The condition of the other two lambs did not aggravate further; these animals were bled on day 8.

Table I

Humoral immune response following vaccination. I
(Efficacy of programmes as compared with base values)

Days after 1st vaccination	Haemagglutination inhibition (HI)			Virus neutralization (VN)		
	Average of $\log_2$ titres	Standard deviation	Significance of differences vs. base values	Average of $\log_2$ titres	Standard deviation	Significance of differences vs. base values
0	3.0	1.0		2.8	2.17	
7*	4.0	1.41	N.S.	2.6	1.82	N.S.
21	5.8	1.1	$P < 0.01$	4.8	0.84	N.S.
35	5.8	0.45	$P < 0.001$	2.4	0.55	N.S.
49	5.0	0.20	$P < 0.05$	1.75	0.90	N.S.
0	3.0	1.58		2.75	1.50	N.S.
10*	3.6	1.34	N.S.	2.67	0.58	N.S.
24	6.6	2.07	$P < 0.005$	3.6	1.82	N.S.
38	6.2	1.64	$P < 0.02$	4.0	1.22	N.S.
52	4.4	0.89	N.S.	2.5	1.00	N.S.
0	2.0	0.70		1.8	0.84	
14*	4.0	0.71	$P < 0.01$	1.75	0.96	N.S.
28	8.0	2.00	$P < 0.001$	9.0	2.12	$P < 0.001$
42	8.0	2.00	$P < 0.001$	6.8	2.05	$P < 0.001$
56	5.8	1.10	$P < 0.001$	5.0	1.0	$P < 0.001$
0	4.0	1.0		3.0	1.22	
21*	3.8	0.84	N.S.	3.0	1.58	N.S.
35	7.2	2.77	$P < 0.05$	6.2	3.03	$P < 0.05$
49	6.8	2.28	$P < 0.05$	5.2	2.39	N.S.

* The day of second vaccination
$t = 2.262 \rightarrow P < 0.05$
N.S. = not significant

Table II

Humoral immune response following vaccination. II
The significance of differences between mean antibody titres found after the second vaccination performed after different intervals

a On day 14 after second vaccination

	Programme	7	10	14	21	Programme	
	7		N.S.	N.S.	N.S.	7	
	10	N.S.		N.S.	N.S.	10	
VN	14	S	S		N.S.	14	HI
		$P < 0.001$	$P < 0.001$				
	21	N.S.	N.S.	N.S.		21	

$n = 5$ $df = 8$ $t = 2.262 \rightarrow P = 0.05$
S = significant N.S. = not significant

b On day 28 after second vaccination

	Programme	7	10	14	21	Programme	
	7		N.S.	S	N.S.	7	
				$P < 0.05$			
	10	N.S.		N.S.	N.S.	10	
VN	14	S	S		N.S.	14	HI
		$P < 0.0001$	$P < 0.001$				
	21	S	N.S.	N.S.		21	
		$P < 0.05$					

$n = 5$ $df = 8$ $t = 2.262 \rightarrow P = 0.05$
S = significant N.S. = not significant

The vaccinated animals and the uninfected controls showed no clinical symptoms.

Necropsy findings of unvaccinated animals included reddening and streakiness (dilation of capillaries) of the mucous membranes of the nasal conchae and the trachea. In the lungs of the lamb bled on day 7 the apical and cardiac lobes had small, dark red, compact (consolidated) areas. In vaccinated animals, no clinical symptoms were observed and, except the medullary swelling of lymph nodes, no macroscopic pathological changes were seen.

Histopathological studies revealed extensive intralobular interstitial pneumonia and mild peribronchial lymphoid cell hyperplasia in the anterior

part of apical and diaphragmatic lobes of the lungs of unvaccinated animals. In the more severely affected lamb, desquamative necrosis of the bronchial epithelium and cellular infiltration of alveoli were also observed in addition to the aforementioned changes. The mediastinal lymph nodes of unvaccinated animals showed hyperplasia of follicles and proliferation of lymphoblasts. In two of the vaccinated animals, the diaphragmatic lobes of the lungs showed intralobular interstitial pneumonia restricted to small areas. Lung lesions were absent in one animal. All three lambs were characterized by pronounced peribronchial lymphoid hyperplasia. The paracortical part of mediastinal lymph nodes showed a pronounced lymphoid hyperplasia while in the cortex only a mild hyperplasia of follicles developed.

The results of re-isolation attempts from nasal swabs and from organs of exsanguinated animals are summarized in Table III.

Table III

Re-isolation of PI-3 virus from lambs

Animals	HI titre at challenge	Virus isolation											
		From nasal secretion after challenge Days								Nasal cavity	From organs		
		1	2	3	4	5	6	7	8		Trachea	Lungs	Lymph nodes
Vaccinated													
1	1:128	+	+	—	—	—	—	—	—	—	—	—	—
2	32	+	+	—	—	—	—	—	—		—	—	—
3	64	+	—	—	—	—	—	—	—	—	—	—	—
Unvaccinated													
4	—<2	+	+	+	+	+	+	+	+	+	+	—	—
5	—<2	+	+	+	+	+	+	—	+	+	+	—	+
6	—<2	+	+	+	+	+	+	Ø	Ø	+	+	+	+
Control													
7	—<2	—	—	—	—	—	—	—	—	—	—	—	—
8	—<2	—	—	—	—	—	—	—	—	—	—	—	—
9	—<2	—	—	—	—	—	—	—	—	—	—	—	—

+ = successful re-isolation
— = unsuccessful re-isolation attempt

Discussion

In a previous work, we brought evidence that our PI-3 virus strains of high HA activity were of considerably better immunogenic potential than those of low HA activity. During inactivation, the ability of strains to induce a hu-

moral immune response (as indicated by both HI and VN tests) underwent an appreciable reduction, parallel with the decrease of HA activity. Since ethylenimine-inactivation, based on nucleic acid alkylation (Primrase and Dimmock, 1980), did not destroy the glycoprotein envelope antigens, there were no changes demonstrable in immunogenicity (see p. 45 in this volume).

Serological studies on calves vaccinated twice at different intervals proved that the curves representing the HI and VN antibodies did not run parallel. A similar phenomenon had been observed in rabbits (see p. 45 in this volume). While HI antibody titres significantly increased after the second vaccination in all cases, the elevation of VN antibody titres was found to be significant only in the group re-vaccinated 14 days after the first vaccination. This is expressed by the significance of HI and VN values found at different times, as compared to the prevaccination values. When comparing the results of vaccination programmes on days 14 and 28 after the second vaccination, the HI test failed to reveal significant differences. As regards the VN antibodies, the re-vaccination given after an interval of 14 days proved to be significantly more effective than that performed after 7, 10 or 21 days (Table II).

Polna and Aleksandrowicz (1975) demonstrated HI antibodies of IgM nature in the early stage of immunity in rabbits inoculated with measles virus. However, in later stages and in convalescent sera of humans only HI antibodies and neutralizing antibodies of the IgG class were found. Presumably, the difference in the appearance of HI and VN antibodies in calves and rabbits after vaccination (see p. 45 in this volume) can be explained in the same way. Further studies are needed for satisfactory clarification of this point.

The lesions developed in the unvaccinated lambs in our challenge experiments were consistent with those described by Hore and Stevenson (1967).

Several authors have studied the efficiency of live and inactivated PI-3 vaccines, in relation to the mode of application. In calves, the attenuated live virus provided better protection after intranasal than after intramuscular application (Gates et al., 1970; Gutekunst et al., 1969; Smith, 1975; Todd, 1973). According to Frank and Marshall (1971), the degree of protection primarily depends on the antibody concentration of the nasal secretion, while the severity and course of the disease on the antibody levels of the serum. It has also been proved that in case of high VN antibody titres in the serum, antibodies might be exudated into the nasal secretion (Marshall and Frank, 1971). Others have reported a definite protective effect of inactivated vaccines in calves (Probert et al., 1978) and lambs (Wells et al., 1978) with high serum antibody titres elicited by the vaccines.

In our studies, the vaccinated and challenged animals failed to develop clinical symptoms, although the lungs of two animals showed mild histopathological changes. While unvaccinated animals were shedding the virus

continuously and virus was also isolated from their respiratory organs, it was not possible to recover the virus from the vaccinated animals. The peribronchial lymphoid hyperplasia found in the lungs and the pronounced hyperplasia of lymphoid cells found in the paracortical part of mediastinal lymph nodes of vaccinated animals after challenge indicated a rapid and pronounced reaction of the cellular immune system. These were not observed in nonimmunized animals. The follicular hyperplasia developed in the cortices of lymph nodes indicated the activity of cells involved in humoral immunity.

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SEROLOGICAL SURVEY ON ROTAVIRAL ANTIBODY IN THE HUNGARIAN SWINE POPULATION BY OPTIMIZED COUNTER-CURRENT IMMUNO-ELECTROPHORESIS (CCIEP)

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552 blood samples collected from 44 large-scale swine breeding farms in 13 Hungarian counties were examined for the presence of rotavirus-specific antibodies by optimized counter-current immuno-electrophoresis (CCIEP). Among the technical conditions influencing the quality and the specificity of the test, the quality of antigen and the type of agarose proved to be decisive.

The antigen was prepared from the faeces of an artificially infected calf by centrifugation, filtration and ultracentrifugation. Its titre ranged from 1:16 to 1:128. The CCIEP was done on microscope slides covered with 4.5 ml of 1% Agarose A (PHARMACIA) gel in barbital buffer of pH 8.6. Electrophoresis was carried out at 7 V/cm for 90 min.

Of the farms examined, 41 (93.2%) proved to be infected by rotavirus. Rotavirus-specific antibodies were demonstrated in 167 blood samples (31.2%). The percentage of seropositive pigs ranged from 6.7 to 100% in the farms surveyed. The prevalence of antibodies in pigs of different ages was 26% in 4 months old pigs, 50.6% in 6 months old pigs, 33.3% in 8 months old and 25.2% in 1 year old or older pigs. The results showed that most of the pigs became infected by rotavirus after the weaning age.

Rotaviruses are presently thought to be one of the major causes of neonatal gastroenteritis throughout the world. Soon after Mebus et al. (1969) reported the presence of rotavirus in the faeces of newborn calves with acute non-bacterial gastroenteritis, rotaviruses have been shown to be associated with neonatal diarrhoea in other mammals including piglets (McNulty et al., 1976; Rodger and Craven, 1975; Woode et al., 1976a), foals (Flewett et al., 1975), lambs (Snodgrass et al., 1976), rabbits (Bryden et al., 1976; Petric et al., 1978), infant mice (Much and Zajac, 1972) and young children (Bishop et al., 1973; Kapikian et al., 1974). Subsequent studies revealed morphological and antigenic relationships among rotaviruses isolated from different animal species. All the known rotaviruses share a common antigen demonstrable by immunofluorescence, complement fixation test, gel diffusion test and immune electron microscopy. Besides, rotaviruses from one species can infect other species (Woode et al., 1976; Thouless et al., 1977).

The detection of rotavirus is still time-consuming and needs special equipment. Their isolation and propagation in cell cultures is also difficult and — except for the bovine isolates — they do not produce a definite cytopathogenic effect. In Hungary, Köves (1979) isolated a cytopathogenic rotavirus from a case of neonatal calf diarrhoea. Others demonstrated rotaviruses by

immunofluorescence or by electron microscopy in newborn calves (Köves et al., 1977; Nagy, 1979) and in piglets (Benyeda and Tuboly, 1978; Nagy and Rátz, 1979).

Probably due to the technical difficulties, only few data have been published on the prevalence of rotaviral antibodies in populations of different animal species including cattle (Acres and Babiuk, 1978; Sato et al., 1981; Schlafer and Scott, 1979; Takahashi et al., 1979; Zygraich et al., 1975), horse (Dagenais et al., 1980; Imagawa et al., 1979; Sato et al., 1981; Takahashi et al., 1979), sheep (Sato et al., 1981; Snodgrass et al., 1977; Takahashi et al., 1979), swine (Sato et al., 1981; Takahashi et al., 1979), dog and cat (Dagenais et al., 1980; McNulty et al., 1978), rabbit (Sato et al., 1981) and, before the present studies, no data were available from Hungary. This study was designed to optimize the counter-current immuno-electrophoresis technique to investigate the prevalence of rotaviral antibodies in swine sera.

Materials and methods

Blood samples were collected in 44 large-scale swine farms located in 13 counties of Hungary. Generally, 10 to 15 animals of different age groups were sampled on each farm. Of the various techniques to detect rotaviral antibodies, counter-current immuno-electrophoresis was selected. The advantage of this test is the rapidity and accuracy to survey blood samples in large number.

Preparation of antigen. Four different antigen preparations were tested. All of them were made from diarrhoeic faeces of rotavirus-infected calves and were prepared as described by Mohammed et al. (1978). Crude faecal antigen consisted of faeces diluted to 1:5 after two cycles of centrifugation at $3000 \times g$ for 30 min to remove the majority of organic material (C antigen). The crude faecal antigen was further clarified by filtration through a 450 nm Millipore filter (F antigen). The filtered antigen was then centrifuged at $100{,}000 \times g$ for 180 min at +4 °C in an MSE SS 50 ultracentrifuge. The pelleted virus was resuspended in phosphate-buffered saline (PBS), pH 7.2, in 1/20 of the original volume (filtered and concentrated, FC antigen). The soluble antigen, present in the supernatant after ultracentrifugation, was used without further concentration (S antigen). The antigens were titrated against the constant dilution (CCIEP titre 1:16) of a calf anti-rotavirus serum.

Counter-current immuno-electrophoresis (CCIEP). The test was done on microscope slides covered with 4.5 ml of 1% gels of various types of agarose products (LITEX HSA, $M_r = -0.13$; PHARMACIA Agarose A and B, $M_r = -0.13$ and -0.25, respectively; SERVA, High EEO, research grade, $M_r = -0.25$) in 0.025 mol/l veronal buffer, pH 8.6, with or without the addi-

tion of 0.1 mg protamine sulphate to one ml of the gel. The wells were punched in three parallel row-pairs. The diameter of the wells was 3 mm and they were filled with 10 μl each. The distances between wells were 2.5 mm. Antigens were placed in the wells facing the cathode and the heat-inactivated sera in the wells facing the anode. The titre of antigen was always adjusted before use to four times concentration of the highest dilution giving definite positive reaction with the standard dilution of the positive control serum. Known positive and negative control sera were included in each run.

The electrophoresis was carried out in the LABOR MIM immunoelectrophoresis chamber 59951 (LABOR MIM, Budapest, Hungary) at room temperature using veronal buffer at 7 V/cm. The ionic strength of buffer varied between 0.025 and 0.075 mol/l, the pH between 8.2 and 8.8, and the time of electrophoresis between 45 and 120 min. The slides were examined for the presence of precipitation lines immediately after the electrophoresis and 24 h later. In the meantime, the slides were kept in a moist chamber at room temperature.

Results

Technical arrangements. From the agarose preparations tested, PHARMACIA Agarose A with an endosmotic value (M_r) of -0.13 was found to be the most appropriate. The other gels showing similar (LITEX HSA, $M_r = -0.13$) or higher electroendosmotic values (PHARMACIA Agarose B and SERVA High EEO, $M_r = -0.25$) gave some reactions or none (Table I). At 7 V/cm, precipitation reactions were complete after 90 min running time. The quality of the result decreased with lengthening the time of run, increasing or shortening the distance between the wells or using a veronal buffer with lower or higher ionic strengths and pH values than 0.05 mol/l and pH

Table I

Comparison of the results of CCIEP performed in 1% gel of different types of agarose products in 0.05 mol/l veronal buffer, pH 8.6

Agarose	Electro-endosmotic (M_r) value	Titre of	
		FC antigen*	Standard positive serum **
LITEX HSA	—0.13	1:4	1:2
PHARMACIA A	—0.13	1:64	1:32
PHARMACIA B	—0.25	0	0
SERVA, high EEO	—0.25	1:4	1:16

* Titrated against the constant 1:4 dilution of a calf anti-rotavirus serum
** Determined with the FC antigen

8.6, respectively. The protamine sulphate content of the gel had no visible effect on the result of precipitation reactions.

For the subsequent testing procedures, CCIEP was performed in 1% gel of PHARMACIA Agarose A using 0.05 mol/l veronal buffer, pH 8.6, under potential difference of 7 V/cm. The time of electrophoretic run was 90 min.

Comparison of various rotavirus antigens. Different dilutions of each of four antigens were compared for sensitivity to detect rotaviral antibodies using the constant 1:4 dilution of a calf anti-rotavirus serum (CCIEP titre 1:64). Table II shows that the filtered and concentrated FC antigen proved to be superior to antigens C and F. No reaction occurred with the unconcentrated S antigen. It was concluded that the FC antigen would be the antigen of choice for routine screening for rotaviral antibodies.

Detection of rotavirus-specific antibodies in swine sera. Out of 44 apparently healthy swine stocks, 41 (93.2%) proved to be infected by rotavirus. Of

Table II

Comparison of various rotavirus antigens for detection of rotaviral antibodies

Test antigen	CCIEP titre*
C antigen	1: 4 to 1: 8
F antigen	1: 4 to 1: 8
FC antigen	1:16 to 1:128
S antigen	0

* Positive end-point dilution of different antigen lots against the constant 1:4 dilution of a calf anti-rotavirus serum

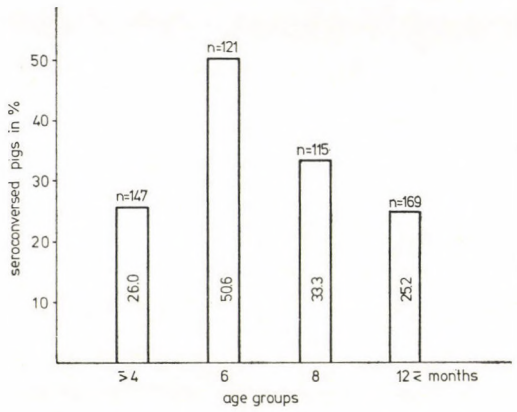

Fig. 1. Distribution of rotaviral antibodies in pigs of different ages

the 552 swine sera, 167 (31.2%) contained rotavirus-specific antibodies. The percentage of seropositive pigs ranged from 6.7 to 100% in the farms surveyed. The distribution of rotavirus-specific antibodies in different age groups is shown in Fig. 1.

Discussion

A variety of technical conditions has been standardized to optimize CCIEP for routine screening of swine sera for rotavirus-specific antibodies. From the study performed, it appears that the quality of antigen and the type of agarose are decisive among the technical conditions influencing the quality and specificity of the test. From the agarose preparations examined, PHARMACIA Agarose A with a moderate electroendosmotic value ($M_r = -0.13$) was found superior to agarose products with higher electroendosmotic values ($M_r = -0.25$), which are generally more suitable for CCIEP.

Among the antigen preparations tested, FC antigen was significantly more sensitive than either the crude or the filtered faecal antigens. Furthermore, the latter two antigens gave multiple lines in the CCIEP, which could be eliminated by purifying of the faecal material by ultracentrifugation. When different lots of FC antigen were reacted with the positive control and test sera, in every case a single precipitation line was observed that needed absorption of sera with faecal or tissue culture rotavirus antigen to be removed.

Results of this serological survey have shown that rotavirus infection is widespread in Hungarian swine stocks. The proportion of the reactors varied in each farm and also in accordance with the age groups. The rate of seroconversed animals increased until six months of age, thereafter it tended to decrease. This indicates that most of the pigs became infected by rotavirus after the weaning age.

For the correlation existing between age and presence of antibody, similar results were reported by Imagawa et al. (1979), who surveyed Japanese light horses for rotavirus antibodies with the complement fixation test. Complement fixing antibodies against rotavirus were found in 15.1% of horses younger than one year of age, 56.3% of two years old and 43.3% of four years old or older horses.

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QUANTITATIVE DETERMINATION OF SOME *FUSARIUM* TOXINS BY GAS CHROMATOGRAPHY

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A method was developed for simultaneous determination of five *Fusarium* mycotoxins: deoxynivalenol, diacetoxyscirpenol, HT-2 toxin, T-2 toxin and zearalenone.

The cereal sample was extracted with ethyl acetate, then with a 6:4 mixture of methanol and water. The extract was purified by Kieselgel 60 column chromatography. The purified sample was reacted with BSTFA (N,O-bis/trimethylsilyl/-trifluoracetamide). The silylether derivatives of the samples were analysed on an SE 52 wall-coated open tubular column. Pure toxins were mixed to the cereal, and recovery and standard deviation tests were performed. Seventy to 80% of the toxin was recovered and the relative standard deviation ranged between 10% and 18%. The method developed was applied to concrete samples.

Feed may often be contaminated by several fungus species, and even the same fungus species may produce several mycotoxins. Therefore, it is an old endeavour to develop an analytical method, suitable for simultaneous detection of more than one toxin in a single analysis. Stoloff et al. (1971) described a method for simultaneous determination of aflatoxin, ochratoxin, zearalenone, sterigmatocystin and patulin. The method was modified by Scott et al. (1972). Roberts and Patterson (1975) reported on simultaneous investigation of 14 toxins. Josefsson and Möller (1977) and Pliszczynszka and Juszkiewicz (1977) were also engaged in the development of so-called multi-mycotoxin detection methods. The method of Gimeno (1979) is suitable for detection of 10, that of Takeda et al. (1979) for detection of 14, mycotoxins. In addition to the authors mentioned, several researchers attempted to solve this problem, however, the methods did not find wide practical application because they require a circuitous purification procedure, and moreover, their sensitivity is poor with respect to some of the toxins. Thus, they do not satisfy the practical demands.

In addition to the thin-layer chromatographic methods mentioned above, Engström et al. (1977) developed a high-pressure liquid chromatographic method for multimycotoxin determination.

In European countries of continental climate, thus also in Hungary, *Fusarium* fungi play an important role in the fungal deterioration of feed quality and in animal diseases caused by mycotoxins. Thus, a toxin detection

method was needed which is suitable for parallel detection of several *Fusarium* toxins (4–6) in a very low concentration range.

In our work, capillary gas chromatography was used for detection of the toxic secondary metabolites most often produced by *Fusarium* fungi.

Materials and methods

a) Vacuum rotadest apparatus (MTA Kutatási Eszközöket Kivitelező Vállalat, Hungary),

b) Block Thermostat (MTA Kutatási Eszközöket Kivitelező Vállalat, Hungary),

c) Packard Model 427 gas chromatograph, equipped with FID detector and HP 3390 A integrator,

d) Screw-cap vials (Pierce, Rockford, Ill. USA),

e) Column glass capillary, drawn on a Hupe Bush capillary drawer manufactured by Hewlett Packard, and wetted according to the method of Grob et al. (1977, 1978).

f) All the solvents used were commercial preparations (Reanal, Hungary), twice distilled before use.

g) Mycotoxin standards: 1 mg/cm³ zearalenone (Supelco 4–6318); 0.5 mg/cm³ T-2 toxin (Supelco 4–6322); 0.5 mg/cm³ diacetoxyscirpenol (Supelco 4–6315); 0.7 mg/cm³ deoxynivalenol; 0.5 mg/cm³ HT-2 toxin (C. J. Mirocha, St. Paul, Minnesota, USA).

h) BSTFA (Pierce, Rockford, Ill. USA).

i) Kieselgel 60 (Merck).

Ten g of wheat was ground to grits fineness. The ground wheat was extracted at room temperature for 2 h with 200 cm³ ethyl acetate, the mixture was repeatedly shaken during extraction. After extraction, the organic solution was filtered off and put aside. The residue was extracted again at room temperature for two hours with 200 cm³ methanol — water mixture (6+4 v/v). The solution was filtered off and the two filtrates were combined. The water content of the solution was removed by 10 g of anhydrous Na_2SO_4, and the solution was evaporated on a vacuum rotadest apparatus. Evaporation yielded an oily liquid residue. This residue was dissolved in 2 cm³ of benzene — acetone (1+1 v/v) mixture and poured on a 10×1 cm column packed with Kieselgel 60. The lipid content of the extract was eluted with 20 cm³ benzene from the column. The mycotoxins tested were eluted with 20 cm³ benzene — acetone (1+1 v/v) mixture. The eluate containing the toxins was evaporated on the vacuum rotadest.

The substance evaporated to dryness was dissolved in 2 cm³ of acetone. Two hundred mm³ of the acetone solution was transferred into a screw-cap

vial and evaporated in N_2 atmosphere. Two hundred mm^3 of BSTFA reagent was introduced into the vial, which was then tightly closed, and heated for 15 min at 60 °C in the block thermostat. The reaction mixture was allowed to cool after the proceeding of the reaction, and 1 mm^3 of the reaction mixture was injected into the gas chromatograph.

Determination by gas chromatography

The column used in the apparatus was drawn from Pyrex glass of 8 mm O.D. and 3 mm I.D. on a Hupe Bush Hewlett Packard drawing apparatus. The deactivation of the glass surface was carried out according to Grob (1977, 1978), while the packing with a statical method. The phase ratio, β = gas volume/wetting volume, was 250.

Column: 14 m, 0.25 mm I.D. wall-coated open tubular column wetted with SE 52
Temperature of thermostat: 180–260 °C, 3 °C/min.
Temperature of injector: 260 °C
Temperature of detector: 260 °C
Carrier gas: H_2, entrance pressure 40 kPa.

Recovery and standard deviation of the method

Recovery and standard deviation values calculated from 11 parallel runs of the 5 substances investigated are shown in Table I for a concentration level of 100 μg/kg.

Results and discussion

Figure 1 shows the chromatograms of standard mycotoxins. Nineteen feed samples were investigated with the method described.

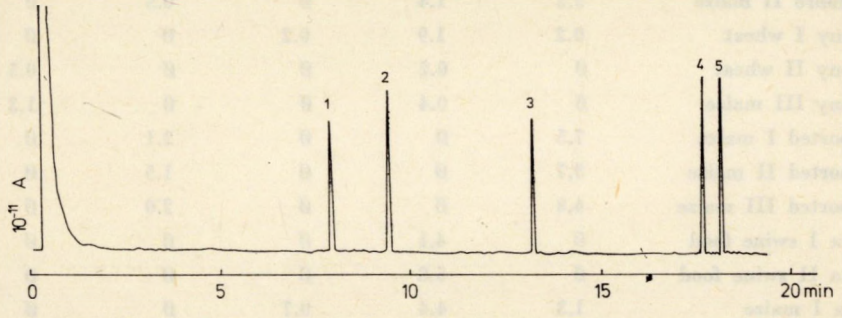

Fig. 1. The chromatogram of standard mycotoxins (1 — deoxynivalenol; 2 — diacetoxyscirpenol; 3 — HT-2 toxin; 4 — T-2 toxin; 5 — zearalenone)

Table I

Recovery and standard deviation values of the five *Fusarium* toxins investigated

Name of toxin	Recovery, per cent	Relative standard deviation, per cent
Zearalenone	78	12
T-2 toxin	67	17
HT-2 toxin	75	13
Diacetoxyscirpenol	83	11
Deoxynivalenol	64	19

The samples selected were presumed to be positive for *Fusarium* toxin on the basis of preliminary veterinary indication. The 7 samples in which no mycotoxin could be detected were not included in the presenting of results. Results are shown in Table II. All the substances investigated could be detected in the samples. In the major part of the samples, more than one mycotoxins were found. This experimental finding supports the hypothesis that in *Fusarium* infection several mycotoxins are formed simultaneously, and syndromes arising in the animals are resultants of the toxic effect of several substances. It happened in certain cases that the effect of a single toxin predominated, and in this case only one predominant toxin could be detected in the test, the quantity of the others was below the limit of detection.

Table II

Test results

Name of sample	Zearalenone, mg/kg	T-2 toxin, mg/kg	HT-2 toxin, mg/kg	Diacetoxy-scirpenol, mg/kg	Deoxynivalenol, mg/kg
Feldebrő I maize	5.7	0.3	Ø	0.8	Ø
Feldebrő II maize	3.2	1.4	Ø	0.5	Ø
Adony I wheat	0.2	1.9	0.2	Ø	Ø
Adony II wheat	Ø	0.2	Ø	Ø	0.5
Adony III maize	Ø	0.4	Ø	Ø	1.3
Imported I maize	7.5	Ø	Ø	2.1	Ø
Imported II maize	3.7	Ø	Ø	1.5	Ø
Imported III maize	4.8	Ø	Ø	2.0	Ø
Pápa I swine food	Ø	4.1	Ø	Ø	Ø
Pápa II swine food	Ø	5.8	Ø	Ø	Ø
Baja I maize	1.3	4.4	0.7	Ø	Ø
Baja II maize	0.7	3.8	0.5	Ø	0.2

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INFLUENCE OF INDUCED MATERNAL ACIDOSIS ON THE ACID-BASE BALANCE OF THE NEWBORN CALF

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The influence of maternal acidosis on foetal and neonatal acid–base status and viability was studied. Lactic acidosis was produced in seven bovine females (five heifers and two cows) by oral administration of a high dose of sucrose in the terminal prepartal period. Two pregnant heifers were used as control.

Follow-up of the main acid–base parameters in blood and urine indicated that short-term (one-day) acute acidosis of the dam did not affect adversely the acid–base status of the calf either in utero or post partum, but subacute acidosis, progressing to a severe condition during 4–7 days of treatment, also involved the offspring; the calves of such dams were either born in acidotic condition, or developed acidosis within a few hours after birth. Two acidotic calves died soon after birth.

The influence of maternal acid-base disturbances on the foetus has been little studied. Observations in humans have suggested that alterations in the mother's acid–base and electrolyte metabolism also affect the metabolic status of the developing human foetus (Thalme, 1967). Pregnant guinea pigs responded to artificially induced hyperventilation by hypocapnia, their foetuses by metabolic acidosis (Morishima et al., 1964). Pregnant rats exposed to hypoxic conditions developed a metabolic acidosis, their foetuses a combined (respiratory-metabolic) acidosis (Thalme, 1967).

Interrelationships between the maternal and foetal acid–base status have been poorly studied in the bovine, although the problem is of great practical importance, since their special digestive system predisposes ruminants to alimentary acidosis.

In the present experiments, we studied the influence of maternal acidosis induced in the terminal stage of pregnancy on the acid–base status, and thereby on the viability of the newborn calf.

Materials and methods

Seven primiparous heifers and two multiparous cows were used in the experiment. The heifers weighed 375–410 kg, the cows 700 and 768 kg. Six heifers and one cow were Hungarian Fleckvieh × Red Pied Lowland crosses, one heifer was a Hungarian Fleckvieh × Holstein–Friesian cross, and one cow was a purebred Hungarian Fleckvieh.

All animals were fed 2–4 kg dairy-cow feed daily, and alfalfa hay ad lib.; the cows of Groups II and III were additionally given sucrose to induce acidosis. The different groups can be characterized as follows:

Group I (control, heifers nos 1 and 2). The foetus was removed by Caesarean section on day 270 of gestation.

Group II (heifers nos 3, 4 and 5). A single 12 g/kg bwt dose of sucrose was administered orally in aqueous solution (about 0.7 kg/l) on day 269 of pregnancy, and 24 h later the calf was delivered by Caesarean section.

Group III (heifers nos 6 and 7; cows nos 8 and 9). Sucrose was administered at 7 g/kg bwt/day dose level, orally in aqueous solution as above, from the 265th day of pregnancy on. The two heifers were subjected to Caesarean section after 4 and 7 days, respectively, of acidogenic diet. Cow no. 8 calved spontaneously after 4 days, the other cow (no. 9) died abruptly on the 3rd day of treatment.

Maternal blood (jugular vein) and urine samples were taken in Group I (control) before the Caesarean section, in Group II 24 h after sucrose administration, and in Group III on each day of acidogenic diet.

Foetal blood samples were withdrawn from the umbilical artery and vein of each Caesarean-derived calf before lifting from the uterus; post-delivery samples were taken from the jugular vein at 0, 10 and 60 min (occasionally at 8, 12 and 360 min) after birth.

The blood samples were withdrawn into heparinized tubes, in anaerobic conditions, and were examined for pH and pCO_2 by a biological microanalyser (OP 210/2, Radelkis, Budapest), and for haemoglobin content by a blood analyser (Ames). The values of the other acid–base parameters, base excess (BE); actual bicarbonate (act. HCO_3^-) were calculated as proposed by Szenci and Nyírő (1981). Urine samples collected by catheterization were examined for pH by the biological microanalyser, and for net acid–base excretion by the method proposed by Kutas (1965).

Results

The acid–base parameters of the control heifers (Group I) were within the physiological range (Eichler–Steinhauff, 1977; Wilson et al., 1977) during the peripartal period. The values determined in the umbilical arterial blood of the foetuses were as follow: pH 7.29–7.34; pCO_2 6.7–7.0 kPa; act. HCO_3^- 23.3–28.3 mmol/l; BE −2.3–+3.2 mmol/l. The acid–base parameters of the newborn calves indicated a shift towards the state of respiratory acidosis during the initial postnatal hours. Ammann et al. (1974) arrived at similar conclusions on examination of the acid–base status of newborn calves delivered by Caesarean section on account of dystocia.

The animals in Group II developed an acute metabolic acidosis during the last hours preceding the Caesarean section (pH 7.23–7.30; pCO_2 5.2–5.5 kPa; act. HCO_3^- −15.9–18.9 mmol/l; BE −5.9––9.6 mmol/l). Drop of the urine pH from 7.8–8.4 to 5.3–5.9, and of the acid–base excretion from +101–+122 to −122––218 mmol/l indicated a rapid development of aciduria. The acid–base parameters determined in the umbilical arterial blood of the foetuses were practically the same as in the control group (pH 7.30–7.35; pCO_2 7.0–7.5 kPa; act. HCO_3^- 24.8–28.0 mmol/l; BE −0.7–+2.1 mmol/l), and foetal respiratory acidosis was also of similar degree. The postnatal development of the viable foetuses was perfectly normal.

In Group III a subacute acidosis was produced by repeated intake of smaller doses of sucrose. The effect of maternal acidosis on the foetal and neonatal acid–base status is described below in greater detail (Tables I–III).

Heifer no. 6 showed a considerable drop of urine pH and a distinct aciduria already on the first day of sucrose feeding. A major drop of blood pH was observed from day 6 on, and severe metabolic acidosis caused a comatose condition on day 7. Analysis of umbilical arterial and venous blood revealed a similar degree of acidosis also in the foetus delivered by Caesarean section on day 7 of acidogenic diet. Exhaustion of the foetal compensation mechanisms led to progression of the acidotic state during the first postnatal minutes

Table I

Parameters of acid–base status
Heifer no. 6

Animal	Time	Blood				Urine	
		pH	pCO_2 kPa	Actual bicarbonate, mmol/l	BE, mmol/l	pH	Net acid–base excretion, mmol/l
Mother	day 0*	7.43	5.9	28.4	4.5	6.9	+ 21
	day 1	7.39	6.2	27.1	2.7	5.6	—165
	day 2	7.35	5.8	23.1	— 1.4	5.6	—160
	day 3	7.35	5.5	22.2	— 2.1	5.8	—163
	day 4	7.36	5.7	22.9	— 1.5	5.9	—165
	day 5	7.35	5.2	20.8	— 3.4	6.0	—137
	day 6	7.31	4.9	17.9	— 6.5	6.4	— 29
	day 7	7.05	4.6	9.3	—18.5	4.7	—
Calf	foetal umbilical artery	7.12	7.0	16.5	—10.9	—	—
	umbilical vein	7.14	6.6	16.1	—11.1	—	—
	1 min after birth	7.08	7.2	15.3	—12.5	—	—
	8 min after birth	6.75	13.8	13.7	—19.1	—	—

* after the first application of sucrose

Table II

Parameters of acid–base status
Heifer no. 7

Animal	Time	Blood				Urine	
		pH	pCO_2 kPa	Actual bicarbonate, mmol/l	BE, mmol/l	pH	Net acid–base excretion, mmol/l
Mother	day 0*	7.39	7.0	30.2	5.3	8.0	+108
	day 1	7.38	6.7	28.8	4.0	6.0	— 55
	day 2	7.35	6.2	24.7	— 0.2	5.3	—136
	day 3	7.23	5.7	17.2	— 8.5	5.3	—169
	day 4	7.16	7.1	18.4	— 8.7	5.0	—129
Calf	foetal umbilical artery	7.37	8.5	35.2	9.4	—	—
	umbilical vein	7.38	8.1	34.3	9.1	—	—
	1 min after birth	7.34	8.4	32.0	6.6	—	—
	10 min after birth	7.27	9.2	30.6	3.6	—	—
	1 h after birth	7.17	11.0	28.7	0.3	—	—
	2 h after birth	7.03	12.0	22.7	— 7.6	—	—
	6 h after birth	6.81	19.3	22.3	—12.4	—	—

* after the first application of sucrose

Table III

Parameters of acid–base status
Cow no. 8

Animal	Time	Blood				Urine	
		pH	pCO_2 kPa	Actual bicarbonate, mmol/l	BE, mmol/l	pH	Net acid–base excretion, mmol/l
Mother	day 0*	7.40	6.0	26.8	2.7	8.4	+139
	day 1	7.31	5.6	20.4	— 4.4	5.6	—138
	day 2	7.25	5.8	18.5	— 7.1	5.7	— 77
	day 3	7.19	5.4	14.9	—11.1	4.9	— 80
	day 4	7.18	5.5	15.0	—11.2	5.6	— 60
Calf	1 min after birth	7.07	7.5	15.7	—12.9	—	—
	10 min after birth	7.08	8.5	18.2	—10.8	—	—
	1 h after birth	7.16	9.5	24.5	— 3.7	—	—

* after the first application of sucrose

and to death of the calf 8 min after lifting from the uterus. The dam was emergency-slaughtered on the same day.

Heifer no. 7 also developed aciduria on the first day of sucrose treatment, as indicated by the values of urine pH and urinary acid–base excretion. The condition progressed to a severe metabolic acidosis by day 4, when Caesarean section was performed. Unlike foetus no. 6, foetus no. 7 showed a compensated alkalosis rather than acidosis, as judged from the parameters determined in umbilical blood samples. However, alkalosis ceased soon after birth, and a respiratory acidosis developed, which led to fatal outcome during the sixth postnatal hour. The dam was killed on the day of the Caesarean section on account of acidotic coma.

Like heifers nos 6 and 7, *cow no. 8* of the same group showed increased acid excretion from the first day of treatment on. The blood parameters indicated development of a non-compensated metabolic acidosis on the third day. Sucrose intake was then stopped, but the acidotic condition tended to progress. Next morning (at 9.00 a.m., on the fourth day) calving commenced spontaneously; the amnion forced itself through the cervix, but failed to burst and the animal showed no indication of labour. Artificial bursting of the amnion at 6 p.m. made the delivery possible by exercising the usual amount of traction. The blood parameters of the newborn calf indicated a distinct metabolic acidosis immediately after birth, and a considerable compensation thereof one hour later; the calf continued to develop normally further on, but the dam died on the day after calving.

Cow no. 9 died spontaneously on the third day of sucrose diet. The blood parameters indicated a severe, non-compensated metabolic acidosis (pH 7.17; pCO_2 6.0 kPa; act. HCO_3^- 15.8 mmol/l; BE −11.0 mmol/l). Examination of the calf was thus not possible.

Discussion

The foetal acid–base balance is regulated indirectly, through the placenta, by the maternal respiratory and renal functions. The H^+ ions and CO_2 released in excess in the course of foetal metabolism are passed for elimination to the maternal circulation, via the umbilical blood. The ovine epitheliochorial placenta is impermeable to the bicarbonate produced by the foetus (Curet, 1970; Baillie et al., 1971), but enables a rapid diffusion of carbon dioxide (Wilson et al., 1977; Kerpel-Fronius et al., 1978).

The acidosis developed in the maternal organism may affect the foetus in two ways. Firstly, under such circumstances hydrogen ions might be transferred in excess from the maternal blood to the foetus. Due to the acidotic load, an increased elimination of hydrogen ions is necessary by the foetal compensatory processes. This situation is further aggravated by parturition,

which involves accumulation of carbon dioxide and lactic acid in the foetal organism, causing thereby a shift of the acid–base status towards the acidic range also in normal conditions. Secondly, maternal acidosis may adversely affect the oxygen and carbon dioxide transfer across the placenta. Reduction of the oxygen supply creates asphyxial conditions in the foetus which can obtain energy mainly by anaerobic glycolysis, at the expense of converting carbohydrate into lactic acid. The accumulation of lactic acid results in metabolic acidosis in foetal blood. According to the present knowledge, maternal acid–base disturbance affects the foetus to a lesser degree than do disorders in the foeto-maternal gas exchange. Foetal acid–base abnormalities usually arise from gas exchange problems, e.g. deficiency of oxygen supply (Gyódi, 1973).

In the present experiments, two different types of acidosis were induced in dams during the terminal stage of pregnancy. Acute acidosis, developed in response to oral administration of a single large dose of sucrose, was moderately severe, and lasted only a day. The acid–base status of the Caesarean-derived calves of such dams did not notably differ from the control; this has indicated foetal ability for in utero compensation of the influence of short-term maternal acidosis. The selective function of the placenta seems to be a major compensation factor, for it can avert the pathological effect of short-term maternal acid–base disturbances (Kerpel-Fronius et al., 1978).

Foetal response was, however, different to a more durable and severe acidotic condition of the dam induced by repeated (4 to 7 day) administration of sucrose during the last prepartal days. In the group so treated, two calves were born with metabolic acidosis, and a third calf developed it within a few hours after birth. Neonatal acidosis soon led to a fatal outcome in two of the three cases, owing to in utero exhaustion of the buffer systems, which thus failed to compensate the acidotic load of respiratory and metabolic origin after birth.

Although the low number of animals used in the experiment does not permit general conclusions, the fact remains that multiparous cows are obviously less tolerant to alimentary acidosis than primiparous animals.

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CORRELATIONS BETWEEN MUSCLE TONE AND ACID–BASE BALANCE IN NEWBORN CALVES: EXPERIMENTAL SUBSTANTIATION OF A SIMPLE NEW SCORE SYSTEM PROPOSED FOR NEONATAL STATUS DIAGNOSIS

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A simple but conclusive new score system, based on a similar scheme used in human obstetrics, is proposed for neonatal status diagnosis in calves. With this new score system, neonatal status is judged by muscle tone, and the cardiac status is also considered in problem cases. The use of muscle tone as sole physical parameter has been justified by close correlations demonstrated between it and neonatal acid–base parameters. This makes possible an immediate precise judgement of neonatal state without laboratory tests, and thereby the immediate application of chemotherapeutic measures whenever required.

Human obstetricians have long recognized that prevention of perinatal infant mortality requires precise knowledge of the newborn's actual status throughout the critical period. Neonatal status diagnosis should thus be based on a system of reliable criteria, which also disclose prognostic information for early postnatal life.

Initially, only the body dimensions, body weight, and certain changes of the central nervous system had been considered as criteria of the neonatal status; the now obsolete terms "asphyxia pallida" and "asphyxia livida" date back to that early period (Lampé, 1973).

During the last 30–40 years, interest has been increasingly focussed on improving neonatal status diagnosis by integration of new criteria based on certain characteristic physical parameters. The first score system of that kind had been pioneered by Lund (1941), but only the next system, proposed 12 years later by Apgar (1953), has become popular all over the world. The so-called Apgar system, named after its originator, is based on the determination of 5 parameters (heart rate, respiration rate, muscle tone, reflex activity, skin colour) one minute after birth.

However, present-day requirements for neonatal status diagnosis are not fully met by the Apgar system either, for it involves a great deal of subjective judgement, which may become a source of errors (Lampé, 1973).

In the early 'sixties, a more objective diagnostic approach was proposed by Saling (1961), who elaborated reliable methods for the assessment of parameters controlling neonatal acid–base balance.

In combination with the traditional score system, follow-up of the acid-base parameters during and after partus enabled an immediate precise judgement of the actual status, and thereby an immediate therapeutic intervention whenever necessary. Berg (1968) observed on comparison of certain life phenomena with the acid–base status interrelationships of the latter above all with muscle tone, next to it with movement, and to a minor degree also with skin colour; the low correlation found with the latter may have been due to difficulties in judging the shade precisely. Based on Berg's findings, Mihály (1973) evolved a simple four-score system, in which solely muscle tone, as the physical parameter showing closest correlation with the acid–base status, is considered as status criterion, and cardiac activity is also judged in problem cases.

Mihály (1973) has used the scores 0 to 3 in the following scheme:

0 — toneless, no heart action
1 — toneless, heart action present
2 — low tonicity
3 — normal tonicity

The above score system is a simple, but efficient, aid for the obstetrician contemplating resuscitation. A simple, practicable score system, enabling neonatal status diagnosis in calves, is greatly needed also in veterinary obstetrics to reduce perinatal losses by immediate application of an adequate drug treatment. The modified Apgar scheme (respiration rate, muscle tone, reflex activity, skin colour) or the blood test-based diagnosis of slight or severe asphyxia (Ammann et al., 1974; Bodenberger, 1979; Maurer-Schweizer and Walser, 1976; Mülling, 1977; Schlerka et al., 1979), which have been hitherto employed for neonatal status diagnosis in calves, proved to be impracticable for field use. To develop a simple new scheme, we examined the four-score system proposed by Mihály (1973) for interrelationships with the acid–base parameters of neonatal calves.

Materials and methods

A total of 147 calves were examined immediately post partum for degree of vitality (V), which was characterized as follows:

V–O: toneless, head drooping, limbs extended, cardiac activity absent
V–I: toneless, head drooping, limbs extended, cardiac activity present
V–II: low tonicity, abdominal recumbency with head requiring support; reduced number and intensity of reflectoric movements
V–III: normal tonicity, head erect, normal reflectoric movements.

After assessment of the V, blood samples were withdrawn from the jugular vein (in V–O cases by cardiac puncture under anaerobic conditions)

Table I

Degree of vitality (V) scores versus mean ($\bar{x} \pm SD$) acid–base parameters in neonatal calves

V scores	n	pH	pCO_2 kPa	pO_2 kPa	Hb mmol/l	BE mmol/l	EBE mmol/l	BB mmol/l	act. HCO_3^- mmol/l	SAT %
V–III	81	7.232	8.7	3.8	7.5	—1.1	0.2	45.7	26.4	32.3
		0.054	1.1	0.6	1.0	2.4	2.4	2.6	2.3	9.2
V–II	47	7.067	10.2	4.1	7.6	—8.6	—6.6	38.1	21.2	28.8
		0.066	1.4	1.0	1.3	3.2	3.2	3.4	2.9	12.0
V–I	14	6.907	11.2	4.1	7.2	—15.5	—13.1	31.0	16.2	24.7
		0.122	1.5	1.5	1.1	4.5	4.1	4.2	2.9	15.1
V–0	5	6.406	29.0	1.9	4.4	—25.0	—22.6	19.6	12.6	3.1
		0.168	12.3	0.7	1.7	4.8	4.4	4.4	3.6	2.4
F	=	279.04	113.87	11.87	12.42	196.85	179.96	203.0	111.1	12.99
	p<	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
Level of signif-	5 %=	0.037	1.3	0.5	0.6	1.6	1.5	1.6	1.4	5.6
icance	1 %=	0.049	1.7	0.6	0.8	2.1	2.0	2.1	1.8	7.4
	0.1%=	0.063	2.1	0.8	1.0	2.7	2.6	2.8	2.3	9.5

into heparinized syringes which were immediately closed airtight by rubber caps and stored among ice cubes until testing. The pH, pCO_2 and pO_2 were determined immediately or within one hour after sampling at the latest, in ABL-1 apparatus (Radiometer, Copenhagen), and biological microanalyser (Type OP 210/2, Radelkis, Budapest), respectively. The haemoglobin concentration was determined with the cyanohaemoglobin test, in a Vitatron haemoglobinometer. With the values of pH, pCO_2, and pO_2 known, the other acid–base parameters (base excess [BE], extracellular base excess [EBE], buffer base [BB], actual hydrogen carbonate content [act. HCO_3^-]) were established by calculation as described earlier (Szenci and Nyírő, 1981). O_2-saturation (SAT) was determined in percentual terms as proposed by Marsoner and Harnoncourt (1976).

The mean value and standard deviation ($\bar{x}$, SD) of each parameter was assessed for each V category, and was compared between categories by analysis of variance.

Results and discussion

The acid–base values assessed for the four V categories and the significance of inter-category variations in acid–base parameters are shown in the attached Table.

The *V–III calves* were born in the state of a slight, mixed respiratory-metabolic acidosis, which can be regarded as physiological. The values of acid–base parameters for the V–III calves corresponded to those published by other authors (Ammann et al., 1974; Bodenberger, 1979; Mülling et al., 1972; Schlerka et al., 1979; Szenci et al., 1980; Walser and Maurer-Schweizer, 1978) as characteristic of vigorous (Apgar 7–8) newborn calves.

The respiratory (pCO_2) and metabolic (pH, BE, EBE, BB, act. HCO_3^-) parameters of *V–II calves* differed significantly from those of V–III ones, and signified over the physiological limit increase of respiratory-metabolic acidosis. Conform to the observation of other authors (Maurer-Schweizer and Walser, 1976), the values of pO_2 and SAT did not differ significantly between V–II and V–III.

The state categorized as V–II corresponded to that generally termed as mild asphyxia (Apgar 4–6) (Ammann et al., 1974; Bodenberger, 1979; Maurer-Schweizer and Walser, 1976; Schlerka et al., 1979). It should be noted that while certain authors (Ammann et al., 1974; Schlerka et al., 1979) observed a significant difference between the pCO_2-values for vigorous and slightly asphyxic calves, others (Bodenberger, 1979; Maurer-Schweizer and Walser, 1976) failed to demonstrate significance.

The *V–I calves* were born with a severe respiratory-metabolic acidosis, with all tested parameters except pCO_2, pO_2 and Hb, differing significantly

($P < 0.001$) from those for V–II. This state corresponded to the one generally termed as severe neonatal asphyxia (Ammann et al., 1974).

The calves classified into the *V–O* category were all stillborn; intrauterine death had been due to exhaustion of the compensation mechanisms. All acid–base parameters assessed in heart blood samples of V–O calves had significantly ($P < 0.001$) reduced values also in comparison to V–I.

In conclusion, the V-score system proposed by us seems to be more practicable in field conditions than either the modified Apgar scheme, or the asphyxia diagnosis based on blood tests. The great advantage of the V system over the latter schemes is that it is simple enough to be mastered, yet fully conclusive of the neonatal state, for the physical criteria used for score evaluation portray the actual acid–base state of the newborn calf without requiring laboratory evidence. This makes possible not only immediate therapeutic intervention if required, but also the differentiation of intrauterine from extrauterine stillbirths. According to our own experience, physical stimulation (cold compress on the nape of the neck, rubbing of the chest) and chemotherapeutic stimulation of the respiratory centre (with Dopram–V®) (Szenci et al., 1980) or Respirot® (Köchli, 1969) after clearing the airways are sufficient for normalization of the neonatal status in V–II cases. V–I calves should additionally receive 5–7 ml/kg buffer infusion (1 mol/l $NaHCO_3$ or 70 g/l Tris-buffer (THAM) and 0.28–0.56 mol/l glucose, as well as strophanthin (Mülling et al., 1972; Walser and Maurer-Schweizer, 1978) for cardiovascular stimulation. Exhaustion of the compensation mechanisms in V–I and to a certain degree also in V–II cases results in late mobilization and a delay in colostrum intake, which predisposes the neonatal calf for *E. coli* enterotoxaemia. Without immediate drug treatment, only a minor part of such calves can be saved by time- and work-consuming intensive care (pail-feeding every other hour).

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PERINATAL CALF LOSSES IN LARGE CATTLE PRODUCTION UNITS

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Two-year stillbirth statistics of 163 large cow herds managed in the cattle production units of 59 state farms were evaluated by analysis of variance or the chi-square test for differences between breeds (Tables I, II), months and years (Table III), primi- and pluriparous dams (Table IV), male and female calves (Table V), levels of milk production (Table VI), herd sizes (Table VII), levels of attendance at calving (Table VIII), levels of training of attendants in charge for night duty, and on Sundays and bank holidays (Table IX), housing conditions for parturient cows, i.e. calving practice (Table X) and management practice during the dry period (Table XI).

Stillbirth incidence varied greatly with the breed, was greatest during the cold season, and lowest during early and late summer. It was generally significantly greater among the primiparous than the pluriparous dams. Male stillbirths occurred significantly more frequently than female stillbirths. Stillbirth incidence tended to increase with the level of milk production. No significant correlation was found between stillbirths and herd size, level of attendance to parturient cows, and housing conditions during calving. The management practice employed during the dry period had, however, a significant impact on subsequent stillbirth incidence.

The profitability of cattle production is decisively influenced by the number of calves born alive and reared to adulthood. The incidence of perinatal losses (in utero and during the first 24 h of extrauterine life) is still as high as 4–7% (Anderson and Bellows, 1967; Bodai, 1978; Greene, 1979; Grommers et al., 1965; Helmig-Schuman, 1964; Rodolph, 1970; Szenci et al., 1981; Voelker, 1967), and covers about a half (50.8% according to Koger et al., 1967, and 47% according to Walser, 1972) of all calf losses. Closer investigations into the factors influencing perinatal calf losses are therefore of immediate interest. An analysis of stillbirth incidence in a large group of intensive cattle production units over a period of two years is reported in this paper.

Materials and methods

The present study covered 163 cattle production units of 59 state farms, in years 1978 and 1979. The total numbers of calvings by primiparous and pluriparous dams of different breeds are shown in Table I. The letter symbols used in Table I for designation of the breeds are also used in the text.

Stillbirth incidence was analysed for distribution between breeds, years and months, heifers and cows, male and female calves, milk yields per lacta-

tion, herd sizes, staff size, training levels of attendants in charge during night shifts, Sundays and bank holidays, calving house constructions, and management practices applied during the dry period.

The data were evaluated by analysis of variance or chi-square test.

Results

1. *Stillbirths in different breeds*

Heifers. Stillbirth incidence was the highest (12.3%) in the lowland breeds (*fg*), the lowest (3.7%) in the Hungaro–Friesian hybrid breed (*e*), and ranged between 5.9–10.4% in the other breeds.

Table I

Absolute number of calvings by primiparous and pluriparous dams within different breeds

	Breed									Total
	a	*b*	*c*	*ac*	*d*	*e*	*fg*	*h*	*i*	
Primiparous dams										
1978	588	2426	4861	6571	1485	1930	526	1748	359	20,494
1979	1231	2127	5492	5502	2476	1660	752	938	694	20,872
Total	1819	4553	10,353	12,073	3961	3590	1278	2686	1053	41,366
Pluriparous dams										
1978	2511	4003	10,460	12,360	3431	5385	2655	3061	2371	46,237
1979	2804	6361	12,056	14,447	5360	5316	2365	3160	1904	53,773
Total	5315	10,364	22,516	26,807	8791	10,701	5020	6221	4275	100,010

a Hungarian Red Pied (HRP), *b* Holstein–Friesian (HF), *c* HRP×HF, *ac* HRP, HRP×HF, *d* HRP, HRP×HF, HRP×RDL (Red Pied Lowland), *e* Hungaro–Friesian hybrid, *f* Black Pied Lowland (BPL), *g* BPL×HF, *h* HRP×RPL, *i* HRP, HRP×BPL, HRP×HF

Cows. Maximum (7.0%) and minimum (3.1%) incidences were associated with the same breeds as above; in the other breeds, mean incidences of 3.8–5.7% were found.

Analysis of variance indicated a significant ($P < 0.001$) inter-group variation in both the heifer and cow category (Table II).

Other authors (Koger et al., 1976; Lindhé, 1967; Elleby and Myqind-Rasmussen, 1971; Laster and Gregory, 1973; Lindström and Vilva, 1977) also observed significant differences in stillbirth incidence between breeds, mainly between heifers of different breeds.

Table II

Stillbirth ($\bar{x} \pm SD$) in different breeds

	Breed										
	a	*b*	*c*	*ac*	*d*	*e*	*fg*	*h*	*i*	F	Least significant difference
Stillbirths of primiparous dams (%)	6.6	8.6	7.5	6.4	6.2	3.3	12.8	6.2	10.1	5.47	5 % = 3.25
										P < 1	% = 4.32
1978	3.6	1.9	2.2	1.9	3.0	1.2	6.5	1.7	8.1	0.001	0.1% = 5.57
Stillbirths of primiparous dams (%)	5.8	7.6	7.6	6.2	5.7	4.2	11.9	6.9	10.7	6.42	5 % = 2.72
										P < 1	% = 3.61
1979	3.2	2.6	1.8	2.0	2.2	2.2	6.5	2.4	4.5	0.001	0.1% = 4.66
Total	6.2	8.1	7.5	6.3	5.9	3.7	12.3	6.5	10.4	12.23	5 % = 2.04
										P < 1	% = 2.69
	3.3	2.2	1.9	1.9	2.5	1.7	6.3	2.0	6.4	0.001	0.1% = 3.45
Stillbirths of pluriparous dams (%)	4.7	5.5	3.9	5.1	5.3	2.7	7.3	3.1	5.6	17.34	5 % = 0.94
										P < 1	% = 1.25
1978	1.4	0.9	0.9	0.9	1.7	0.6	1.5	1.0	1.2	0.001	0.1% = 1.61
Stillbirths of pluriparous dams (%)	5.4	5.4	3.7	4.7	4.5	3.5	6.8	5.3	5.8	6.64	5 % = 1.12
										P < 1	% = 1.49
1979	1.7	1.2	0.7	0.7	1.5	0.8	2.3	1.4	1.4	0.001	0.1% = 1.92
Total	5.0	5.4	3.8	4.9	4.9	3.1	7.0	4.2	5.7	18.21	5 % = 0.73
										P < 1	% = 0.97
	1.5	1.0	0.7	0.8	1.6	0.8	1.9	1.6	1.2	0.001	0.1% = 1.24

2. *Monthly and annual fluctuation of stillbirth incidence*

Heifers. Stillbirth incidence was the greatest during the first three months of the calendar year in both years, then it tended to decrease gradually until it fell to a minimum ($4.7 \pm 3.3\%$ in 1978, and $5.6 \pm 2.1\%$ in 1979) in September. A rise followed during the last quarter of the year, showing a peak in September (9.3%) and another in December (8.7%) in 1978, and a more even rise in 1979.

Cows. The monthly and annual distribution of stillbirth incidence was almost identical in the two years studied. A gradual decrease beginning in January was followed by an increase in May and by another decrease to a level at which stillbirth incidence persisted for the rest of the year.

Analysis of variance revealed significant ($P < 0.01$; $F = 2.66$) inter-month variations in the heifer category, in which stillbirth incidence was significantly lower from April to September than in February ($P < 0.01$) and March ($P < 0.05$), and also significantly ($P < 0.05$) lower in January, November and December than in February (Table III).

Table III

Monthly distribution of stillbirth incidence ($\bar{x} \pm SD$) in primiparous and pluriparous dam groups

Months	Jan.	Feb.	March	April	May	June	July	August	Sep.	Oct.	Nov.	Dec.
Stillbirths of primiparous dams (%)	9.3	8.1	8.8	7.7	7.5	7.4	4.7	5.9	9.3	5.4	7.2	8.7
1978	6.8	6.2	4.3	3.8	4.6	2.7	3.3	2.8	8.3	1.9	4.1	2.4
Stillbirths of primiparous dams (%)	7.5	11.0	10.4	5.9	6.3	6.7	5.6	5.9	6.3	7.0	7.9	7.1
1979*	2.6	6.7	3.7	2.5	2.7	3.0	2.1	2.8	2.4	4.9	4.3	3.7
Total**	8.4	9.5	9.6	6.8	6.9	7.0	5.1	5.9	7.8	6.2	7.5	7.9
	5.1	6.4	4.0	3.2	3.7	2.8	2.7	2.7	6.1	3.7	4.1	3.1
Stillbirths of pluriparous dams (%)	5.4	5.2	5.4	4.6	5.7	4.3	4.4	4.1	4.5	4.8	4.6	4.6
1978	2.1	1.6	1.9	1.6	2.3	1.9	1.2	1.3	1.8	1.4	1.8	1.6
Stillbirths of pluriparous dams (%)	5.4	5.5	5.0	4.6	5.5	4.7	5.0	5.3	5.2	5.3	4.0	5.2
1979	1.6	1.1	1.8	1.7	2.5	1.1	2.0	2.3	2.2	1.0	0.7	1.8
Total	5.4	5.3	5.2	4.6	5.6	4.5	4.7	4.7	4.8	5.0	4.3	4.9
	1.8	1.3	1.8	1.6	2.3	1.5	1.6	1.9	1.9	1.2	1.3	1.6

*$F = 2.66$ ($P < 0.01$), least significant difference $0.05\% = 2.97$; $0.01\% = 3.91$; $0.001\% = 5.04$

** $F = 2.58$ ($P < 0.01$), $0.05\% = 2.35$; $0.01\% = 3.1$; $0.001\% = 3.97$

Lindhé (1967), Auran (1972), as well as Lindström and Vilva (1977) reported a significant increase in stillbirth incidence in both primiparous and pluriparous dams during the summer months, whereas Philipsson (1976) found no significant seasonal differences.

3. *Stillbirths of heifers and cows*

The chi-square test was used to compare the numbers of live-born and stillborn calves between primiparous and pluriparous dams within breed categories. Stillbirth frequency was significantly greater among heifers than among cows in all breeds except *a*. The significance of difference was at the $P < 0.001$ level for 6 of the 9 breeds (Table IV).

Similar significant differences were reported in heifer vs. cow relation by Van Dieten (1963), Anderson and Bellows (1967), Koger et al. (1967), Lindhé (1967), Auran (1972), Walser (1971), Laster and Gregory (1973) and Remmen (1976).

Table IV

Absolute number of live-born and stillborn calves in primiparous and pluriparous dam groups

Breeds	Primiparous dams		Pluriparous dams		P <
	live-born	stillborn	live-born	stillborn	
a	1,706	113	5,038	277	n.s.
b	4,188	365	9,794	570	0.001
c	9,569	784	21,648	868	0.001
ac	11,302	771	25,470	1337	0.001
d	3,722	239	8,366	425	0.01
e	3,450	140	10,361	340	0.05
fg	1,125	153	4,661	359	0.001
h	2,509	177	5,960	261	0.001
i	944	109	4,026	249	0.001
Total	38,515	2851	95,324	4686	0.001

4. *Sex distribution of stillbirths*

Female calves. Considering primiparous and pluriparous dams, stillbirth incidence was the lowest in breeds *e* (3.2%), and *c*, *e* (2.9%; 3.1%), and the highest in breeds *i* (8%) and *a*, *b* and *fg* (4.6–4.8%), respectively. In the remaining breeds, stillbirth incidences of 4.3–6.4% and 3.4–4.3% were established for the female calves of primiparous and pluriparous dams, respectively.

Male calves. The minima and maxima of male stillbirths were associated with the same breeds in both the heifer and the cow category, the lowest incidences (4.6%; 3.3%) having been found in breed *e*, the highest ones in breeds *fg* (17.5%; 9.4%). In the other breeds, incidences of 6.3–12.7% and 4.8–7.3% were found for the male offspring of primi- and pluriparous dams, respectively.

The sex ratio was also established for the offspring groups of heifers and cows; the percentage of male calves was 50.4 and 50.6%, respectively. A similar sex ratio was found by Lindström and Vilva (1977).

We demonstrated by the chi-square test no significant difference in stillborn incidence between male and female offsprings of *d* and *h* primiparous dams, and *a* and *e* pluriparous dams. In the other breeds, the differences were at various levels of significance.

Consistent observations were made earlier by Woodward and Clark (1959), Van Dieten (1963), Arthur (1966), Anderson and Bellows (1967), Walser (1972) and Laster and Gregory (1973), whereas Lindström and Vilva (1977) found a significant difference exclusively in the heifer category.

Table V

Sex distribution of stillbirths

Breeds	Primiparous dams		Sex ratio ♀/♂	P <	Pluriparous dams		Sex ratio ♀/♂	P <
	Male	Female			Male	Female		
	stillbirth per cent				stillbirth per cent			
a	4.3	8.1	51.2	0.01	4.8	5.6	51.2	n.s.
b	6.4	9.7	49.3	0.001	4.6	6.5	49.5	0.001
c	6.1	9.0	50.6	0.001	2.9	4.8	50.6	0.001
ac	4.7	8.0	50.4	0.001	3.9	6.0	50.5	0.001
d	5.7	6.3	49.9	n.s.	4.1	5.5	51.0	0.01
e	3.2	4.6	50.9	0.05	3.1	3.3	50.1	n.s.
fg	6.2	17.5	51.0	0.001	4.7	9.4	51.6	0.001
h	6.0	7.2	50.5	n.s.	3.4	5.0	50.8	0.01
i	8.0	12.7	50.1	0.05	4.3	7.3	51.2	0.001
Total	5.4	8.4	50.4	0.001	3.9	5.9	50.6	0.001

5. *Interrelationship between stillbirth incidence and milk production*

Stillbirth incidence was practically identical in the cow herds producing 3501–6000 kg milk per annum (87% of the herds covered in the study).

The greatest incidence (7.87%) was found in the herds with an annual lactation yield above 6000 kg, and the lowest incidence (3.14%) in those producing 3000–3500 kg milk.

Table VI

Interrelation between stillbirth rate and annual milk production

Production groups	Milk production kg/year	n*	Stillbirth per cent	F	Least significant difference
1	3000–3500	22	3.14±1.83		
2	3501–4000	65	5.4 ±3.13		
3	4001–4500	110	4.94±2.93	4.37	lsd (0.05) = 1.06
4	4501–5000	67	4.89±2.14	(P < 0.001)	lsd (0.01) = 1.40
5	5001–5500	42	4.86±2.64		lsd (0.001) = 1.79
6	5501–6000	11	5.28±1.44		
7	>6000	9	7.87±2.73		

* number of herds

Analysis of variance disclosed significant ($P < 0.001$; $F = 4.37$) differences between the production groups (Table VI). The lowest incidence (3.14%) differed from incidences in the other groups, except groups no. 3 and 4, at the $P < 0.01$ level of significance, and the highest incidence (7.87%) increased still more significantly ($P < 0.001$) over the other values.

6. *Relationship between stillbirth incidence and herd size*

Table VII

Relationship between stillbirth rate and herd size

Herd size	n*	Stillbirth per cent	F
→100	13	5.5±3.5	
101–200	36	4.5±3.3	
201–300	67	5.3±2.8	
301–400	74	4.9±2.6	
401–500	38	5.4±3.1	n.s.
501–600	35	5.5±2.5	
601–700	30	5.5±2.2	
701–900	17	5.2±2.2	
901→	16	4.9±1.9	

* number of herds

This study covered 326 herds (Table VII). Variance analysis revealed no significant difference in stillbirth incidence between herds of dissimilar sizes. The lack of significant difference agrees well with the observations of Oxender et al. (1973) and Hartman et al. (1974), who, however, found that stillbirth incidence tended to increase with the herd size (10.5% and 12.1%, respectively, for sizes of >200 and 200—300). According to Speicher and Hepp (1973), there is no relationship between herd size (<25–>70) and calf mortality at birth.

7. *Relationship of stillbirth incidence with staffing*

One trained obstetrics technician was in charge of the calvings in more than 50% of the units studied, no trained attendant was permanently employed in units with small herd sizes (2.5% of the herds), and up to 4 trained technicians belonged to the permanent staff of units with large herd sizes (2.2% of the herds).

Comparison by analysis of variance revealed no significant difference between the differently staffed unit groups, nor was there a significant interrelationship between stillbirth incidence and the number of animals in charge of one person (Table VIII).

Table VIII

Relationship between stillbirth rate and level of attendance

No. of trained attendants	n*	Stillbirth per cent	F
1	191	5.1±2.6	
2	76	5.1±2.5	
3	44	5.3±2.1	n.s.
4	7	4.5±2.5	
0	8	2.1±1.6	

* number of herds

8. *Relationship of stillbirth incidence with the training level of attendants in charge of night duty and attendance on bank holidays*

Duty in the calving house at nights and on Sundays and bank holidays was in charge of the night-man (a), obstetrics technician (in 66% of the cases) (b), or the attendant on-duty was in charge.

Analysis of variance revealed a significant difference in stillbirth incidence only between group 4, which was of small size, and the other groups (Table IX).

Table IX

Relationship between stillbirth rate and level of training of the attendants

Groups	Attendants on duty at night and on Sundays and bank holidays	n*	Stillbirth incidence (%)	F	Least significant difference
1	*a*	27	5.0±3.1		
2	*b*	216	5.3±2.4	4.0	lsd (0.05) = 0.93
3	*ab*	68	4.5±2.5	(P<0.01)	lsd (0.01) = 1.22
4	*c, bc, ac*	15	3.4±2.6		lsd (0.001) = 1.57

* number of herds
a night watchman; *b* obstetrics technician; *c* attendant on duty for the day

9. *Relationship of stillbirth incidence with housing conditions of parturient dams*

In most of the units (86.5%) the dams were conducted over to the calving stalls after the onset of labour (51.8%), or obstetric assistance was rendered in the stand of the calving house (43.7%). Only 1.2% of the units studied lacked special calving premises. We found no significant differences between stillbirth incidences in the housing conditions analysed (Table X).

Table X

Stillbirth rate under different conditions of calving practices (housing)

Housing conditions during parturition	n*	Stillbirth per cent	F
a	23	5.0±2.8	
b	169	4.8±2.4	
c	116	5.3±2.9	n.s.
Other	18	4.4±2.5	

a foal stand; *b* calving stalls; *c* stand in calving house; other: *bc*, *bd*, stand in dairy cow house (*d*: yard)
* number of herds

10. *Relationship of stillbirth incidence with "dry" management practice*

The tying-down system (a_3 in Table XI) was employed during the dry period in 35.6% of the units studied, and the systems designated as a_2, *d* and *db* in Table XII each was employed in more than 10% of the cases.

Table XI

Stillbirths and management practices during the dry period

Management system	n*	Stillbirth per cent (%)	F	Least significant difference
a	31	5.2±3.2		
a_1	29	5.4±2.5		
a_2	58	4.4±2.1		lsd (0.05) = 1.12
a_3	116	4.9±2.8	2.77	lsd (0.01) = 1.48
d	40	4.8±3.0	(P < 0.05)	lsd (0.001) = 1.89
b	15	7.1±2.0		
db	37	5.8±2.4		

a tying-down system; a_1, a_2, a_3 tying-down system with exercise (yard, pasture) over 2–3 seasons of the year; *b* tying-down system with daytime exercise in the yard; *d* loose housing system
* number of herds

Stillbirth incidence was the lowest (4.4–4.9%) after management under the systems a_2, a_3 and *d* during the dry period, and the highest (7.1%) after management in tying-down stalls with daytime access to the yard. Analysis of variance showed significant ($P < 0.05$; $F = 2.77$) inter-group variations. Under the *b* system, stillbirth incidence increased significantly over *db* ($P < 0.05$), a_1 ($P < 0.01$) and *a*, a_2, a_3 and *d* ($P < 0.001$). A significant ($P < 0.05$) increase was also observed in *d* and *b* over *a*.

Oxender et al. (1973) observed an appreciable, but statistically not significant, increase in stillbirth incidence in loose housing conditions (7%) compared to management in tying-down stalls (5.9%).

Discussion

The profitability of intensive cattle production greatly depends on the relative proportion of live-born calves.

In the herds examined in this study, losses from perinatal mortality amounted to more than 33,000,000 Forints (approximately one million dollars) (the average price of a calf was Ft 4500). Thus, reduction of losses from stillbirth contributes to the internal reserves of cattle production.

The bony birth canal of the bovine female offers less favourable conditions for parturition than do the maternal passages in other species, yet the well-managed and well-fed heifer or cow usually does not require human assistance at calving. Lack of exercise and overfeeding account for incomplete dilatation of the maternal passages, and thereby for an increase in stillbirth incidence, whence human assistance is indispensable in such cases.

The survey presented in this report has shown that the frequency of stillbirths is the greatest from late autumn to early spring (Table III), when extreme climatic influences, food loss or spoilage by inadequate storage, and low roughage rations bias the conditions of management and feeding.

Further to these factors, rearing techniques and breeding conditions (body weight, age, and measurements of the pelvis) are also responsible for the significantly greater stillbirth incidence found among heifers vs. cows (Table IV). The significantly greater percentage occurrence observed among male calves vs. female calves can be ascribed to the greater frequency of larger fetal weight and body dimensions in the male sex (Table V).

Excessive fodder rations fed to enhance milk production account not only for economically important metabolic disorders of dams, but also for an increase in stillbirth incidence (Table VI).

Unlike other investigators (Oxender et al., 1973; Hartman et al., 1974), Speicher and Hepp (1973) and we found no appreciable increase in stillbirth incidence either within or between breeds with the elevation of herd size (Table VII).

Inadequate attendance to the parturient animals (at night, on Sundays and bank holidays, or in loose housing conditions) can, however, account for a significant increase in stillbirth incidence (Tables IX and X).

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PERIPARTAL FLUCTUATIONS OF PLASMA AND HEPATIC LIPID COMPONENTS IN DAIRY COWS

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During calving, healthy dairy cows showed a significant decrease in plasma total lipid (TL) and total cholesterol (TCh) relative to the pre- and postparturient concentrations. The average decrease in plasma TL was assessed as 35%.

The free fatty acid (FFA) level increased to about three times the initial value during calving, on the average to 4.5% against the pre- and post-parturient range of 1.1–1.8%.

The hepatic TL of the parturient cow increased over 1.5-fold of the preparturient level, and returned to approximately preparturient level by 2 months post partum.

An analysis of the fatty acid components of plasma and hepatic lipids showed the presence of the same 12 components in both; of these, C16:0, C16:1, C18:0, C18:1, C18:2 and C20:4 showed significant fluctuations, whereas C12:0, C14:0, C17:0, C18:3, C20:0 and C22:0 did not change significantly in the peripartal period.

C18:0, C18:1 and C18:2 made up 85–90% of the plasma lipids, and C16:0, C18:0 and C18:1 70–80% of the hepatic lipids.

Despite diversities in the percentage participation of identical fatty acid components in plasma and hepatic lipids, the respective changes were similar. Simultaneous monitoring showed that the most characteristic changes in plasma fatty acids were soon followed by consistent alterations in the liver. The changes in the relative proportions of fatty acids were greater in the liver, and the standard errors of the mean values were also greater. During calving, the plasma and hepatic lipids equally showed C16:0, C16:1 and C18:1 concentration increase, and C14:0, C18:0, C18:2 concentration decrease.

The fluctuations observed in the parameters of lipid metabolism in both plasma and liver indicate that during calving dramatic, yet physiological changes, comparable to those observed in fasting or ketotic condition, occur in the lipid metabolism of the healthy cow, and may give rise to diagnostic confusion, if misinterpreted.

The lipid metabolism of ruminants differs from that of non-ruminants in several respects. Pathological alterations in the ruminant lipid metabolism are responsible for performance-limiting conditions, above all for peripartal metabolic disorders, which give frequently rise to serious diseases such as the fatty liver syndrome and ketosis.

Follow-up of changes in certain parameters of the lipid metabolism can not only inform on the pathogenesis of the above diseases, but can also enable their early diagnosis during the subclinical stage, and the assessment of dietary energy levels.

For this reason, the lipid metabolism of dairy cows has been extensively studied in both normal and pathological conditions. The survey of literary data below will be restricted to observations related to our own investigations.

The total lipid (TL) concentration of the blood plasma of dairy cows varies between 2 and 6 g/l, depending on the breed and the stage of lactation

(Rindsig and Schultz, 1974). Wallenius and Whitchurch (1979) established mean TL values of 4.24, 5.26 and 5.29 g/l for days 33, 89 and 139 of lactation, respectively.

Eighty-five to 90% of the TL is composed of approximately equal amounts of phospholipids (PL) and total cholesterol (TCh = free plus esterified cholesterol) (Storry and Rook, 1964). Seventy-five to 90% of the TCh appears in the blood in an esterified form. In normal conditions triglycerides (TG) cover less than 10%, free fatty acids (FFA) less than 5% of TL (Pehrson, 1971). The TG concentration is more than twice greater in dry than in lactating cows, whereas the reverse is true for FFA (Henricson et al., 1977).

The plasma TG level of the cow is low immediately post partum, 0.17–0.18 mmol/l according to Hartmann and Lascelles (1965), and 0.09–0.13 mmol/l according to Bickerstaffe et al. (1974). It has even been suggested that occasionally no detectable amount of TG was present in the plasma of lactating dairy cows (Raphael et al., 1973).

The fact that marked alterations take place in bovine lipid metabolism during the peripartal period had originally been recognized by Maynard and colleagues as early as in 1931. The plasma FFA and PL rise markedly during the last prepartal days and the first postpartal week, then they tend downwards as lactation progresses. The plasma FFA and PL levels of lactating cows increase over those of dry cows and male bovines. According to Lennon and Mixner (1957), the TCh is significantly lower at calving than either before or after it; Rindsig and Schultz (1974) believe that changes in TCh do not involve appreciable alterations in the relative proportions of free and esterified cholesterol (Ch).

The plasma TL level of the cows, examined by Duncan and Garton (1963) dropped to a minimum at calving, and began to rise with the onset of lactation. The concentration changes did not appreciably affect the relative proportions of the main lipid components. No major fluctuations occurred in the fatty acid composition of plasma TG, PL and FFA during gestation, calving and lactation. The linoleic acid component of the Ch-esters increased over the linolenic acid component during pregnancy, but not during calving and lactation.

Yamdagni and Schultz (1970) observed drop of TL to about two thirds of the normal mean value in cows diseased in ketosis. That change involved a quantitative decrease in all components of TL except FFA, which tended to increase. In ketosis, all plasma lipid fractions except FFA showed a change in fatty acid composition, in that the amount of palmitic and oleic acid increased, while that of stearic acid decreased; a reduction in the linoleic acid component occurred mainly in PL and Ch-esters.

Opinions have been divergent on the ketosis-associated increase in FFA. According to Adler et al. (1963) plasma ketone and FFA levels change in-

dependently of one another. Kronfeld (1965) has suggested that increase in the plasma FFA of lactating cows indicated increased lipid mobilization, and could thus be utilized as a clinical diagnostic parameter, although the concentration increase of FFA was low in certain cases of ketosis. Against this, Radloff and Schultz (1967) demonstrated a considerable increase in the plasma FFA level already in the early stages of ketosis.

Fasting cows (starved for 6 days) showed an about fivefold increase in plasma FFA, decrease in the TL, PL and TCh, as well as low stearic acid, but high oleic acid content of plasma FFA and TG (Brumby et al., 1975).

The percentual fatty acid composition of plasma TL was established in calf blood as follows: C16:0, 17.3; C16:1, 4.8; C18:0, 15.6; C18:1, 25.8; C18:2, 32.0; C18:3, 2.6; C20:3, 0.1; C20:4, 2.2 (Noble et al., 1975).

A minor to major degree of lipid accumulation always takes place in the liver of dairy cows during the first postpartal week (Reid et al., 1977). The reason is in all probability the endocrinologically controlled increased mobilization of lipids from the adipose tissues, in compensation of the negative energy balance characteristic of early lactation (Reid et al., 1979).

The lipid content of the liver can be investigated morphologically, by stereological methods, when the fractional volume of the liver cell occupied by fat droplets is determined. The results are expressed as μm^3 fat/100 μm^3 liver cells. By biochemical approach, appropriately extracted lipid content is measured in terms of g/kg wet liver mass. Biochemical determination yields lower values than stereological measurement.

The stereologically and biochemically established values show a low correlation in respect of TL, a close correlation in respect of TG, and no significant correlation in respect of the other hepatic lipid fractions (Collins and Reid, 1980).

Collins and Reid (1980) demonstrated by biochemical analysis an almost threefold increase in hepatic TL during the first postpartal week relative to the value found 8 weeks before calving. The bulk of the increase was due to a greater hepatocellular synthesis of TG, which increased 23 times over the starting value. An appreciable increase was also found in the hepatic levels of the "solvent front" (SF) and FFA, whereas the relative amounts of PL and TCh tended to decrease. The PL was still fairly low 8 weeks after calving, whereas the other hepatic lipid fractions showed practically the same relative proportions as before calving.

In fasting cows, Brumby et al. (1975) observed duplication of hepatic TL, which covered a threefold increase in both TG and FFA, and a 20% increase in TCh. The deposition of TG has been attributed to an increased hepatocellular FFA uptake, which was disproportionally greater than the hepatic lipoprotein synthesis. Reid et al. (1977) attributed the accumulation of TG to a decrease in hepatic TG-mobilization.

Our own experiments, description of which is forthcoming, were performed to obtain information on pre- and postparturient changes in the lipid metabolism of healthy dairy cows, by simultaneous monitoring of the main lipid fractions and fatty acid components of TL in plasma samples and liver biopsies over three months before and two months after calving.

Materials and methods

Ten clinically healthy cows, all Holstein-Friesian × Hungarian Fleckvieh crosses, were used after their first to third lactation. There was little difference between the body mass and milk production (5200–5800 kg per year) of the animals, and all were pregnant at roughly the same period of time.

The experiments were started 3 months before the expected calving term, and were continued for 2 months post partum, i.e. they lasted from October to March. The pre-calving diet contained, in addition to the maintenance ration, nutrients ensuring even development of the fetus and, uniformly, 16 kg daily milk production. During lactation, the diet was adjusted to the needs established from test milkings. The basal diet consisted of high-quality maize silage and lucerne hay, and concentrate was added to it as production ration.

The cows showed no symptoms of clinical illness during the 5-month period of study. Each delivered a healthy calf. Monthly analyses of blood, urine and ruminal content indicated no change either. Light microscopic examination of liver biopsy specimens revealed presence of lipid vacuoles in most hepatocytes during the calving period. The vacuoles were relatively large in the centrolobular hepatocytes, and smaller in the peripheral ones. The hepatolobular structure was intact, but occasionally masked by massive vacuolization. The hepatocellular nuclei were also intact. Little hepatocellular lipid, if any, could be detected after the first postpartal month, and the microscopic appearance of the liver was in every respect normal during the second month. The cows were under observation until the next calving; optimal management and feeding conditions were maintained throughout, and fertilization and calving took place on term. Blood samples were withdrawn from the jugular vein, and liver biopsies of about 0.1 g were taken by percutaneous (Vim-Silvermann) needle, at the beginning of the experiment and subsequently at monthly intervals, on 6 occasions altogether, always after the morning feeding.

The biopsy specimens were processed within 24 h as follows. Hepatic TL was extracted in 2:1 chloroform–methanol, the crude extract was thoroughly shaken with 0.9% NaCl, added in equal volume to the solvent and, after partition of the phases, the bottom phase was collected and evaporated in a water bath at 50 °C, in N_2-atmosphere. The mass of the dry residue, expressed

in per cent related to wet liver tissue, gave the hepatic TL concentration (Folch et al., 1957).

The extracted hepatic lipids were transformed to methylesters with methanol, in the presence of sulphuric acid as catalyst, and were analysed for fatty acid–methylester composition by gas chromatography in a CHROM 42 apparatus, using a 1.8 m long glass column with 3 mm inner diameter, packed with 100–120 mesh GAS–CHROM–Q,* moistened with 10% HI–EFF–1BP.* An isothermic column temperature (180 °C) was maintained, and reference standards prepared from fatty acid–methylester preparations* supplied with the apparatus were used for identification of the fatty acid peaks. The quantitative determination of the fatty acid–methylester content of the samples was based on measurement of the areas beneath the peaks representing the different components, and the results were expressed in percentual terms.

In the plasma samples TL, TCh and FFA content were determined by the methods of Zöllner and Kirsch (1962), Wattson (1960) and Duncombe (1964). The fatty acid composition of the TL was analysed as above, using extracts of 5 ml blood plasma for transformation to fatty acid–methylesters with methanol, in the presence of sulphuric acid. Gas chromatographic analysis, too, was performed in the same conditions as above.

Results

a) *Plasma analysis*

The *TL, TCh and FFA concentrations* found in the blood plasma of cows during the last 3 months of pregnancy and the first 2 months of lactation are shown in Table I.

Table I

Peripartal fluctuations in the plasma TL, TCh and FFA concentration of dairy cows ($\bar{x} \pm s$)

Sampling time	Total lipid (TL) g/l	Total cholesterol (TCh) mmol/l	Free fatty acid (FFA) mmol/l	FFA% TL = 100
Month –3	$4.6^{ae} \pm 1.1$	$4.9^{a} \pm 2.2$	$0.18^{a} \pm 0.06$	1.1
,, –2	$4.8^{b} \pm 1.2$	4.8 ± 2.9	$0.18^{b} \pm 0.03$	1.1
,, –1	$3.6^{df} \pm 0.8$	$3.2^{ad} \pm 1.6$	$0.15^{c} \pm 0.02$	1.2
CALVING	$3.1^{abc} \pm 0.7$	$3.3^{b} \pm 1.0$	$0.49^{abcd} \pm 0.04$	4.5
Month +1	$5.1^{a\,c} \pm 1.5$	$4.1^{c} \pm 1.4$	$0.33^{abc} \pm 0.12$	1.8
,, +2	$6.5^{adef} \pm 2.5$	$5.8^{bcd} \pm 1.5$	$0.26^{d} \pm 0.2$	1.1

a, b, c, d identical letters indicate significant ($P < 0.05$) difference between values so marked within a category; *e, f* as above, but significant at $P < 0.001$ level

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The TL and TCh content of the plasma decreased significantly ($P < 0.001$; $P < 0.05$) during the last prepartal month, and especially at calving, relative to the values found in the first two months of the experiment. The low level of TL at calving deserves special mention. Both TL and TCh increased significantly ($P < 0.001$; $P < 0.05$) after calving.

The plasma FFA level increased significantly ($P < 0.001$) (to $\bar{x} = 0.49$ mmol/l) over the prepartal value ($\bar{x} = 0.15$–0.18 mmol/l) during the period of calving, and tended to decrease ($P < 0.001$) after it, but at 2 months post partum it was still considerably higher than before calving.

The relative percentage of FFA in TL showed an appreciable increase (4.5%) over the usual range (1.1–1.8%) only during the period of calving; the reason is that the postpartal values of TL and FFA increased to similar degrees over those found in dry cows.

The relative proportions of the fatty acid components of plasma lipids showed distinct and characteristic changes during the peripartal period.

Gas chromatographic analysis detected 12 fatty-acid components, of which the following six showed significant ($P < 0.05$) or highly significant ($P < 0.001$) alterations in percentual contribution between samples withdrawn at different times during the period of study:

palmitic acid (C16:0)
palmitoleic acid (C16:1)
stearic acid (C18:0)
oleic acid (C18:1)
linoleic acid (C18:2)
arachidonic acid (C20:4)

The other six fatty acids were detected in the blood plasma in all the samples, but did not show significant changes between samples:

lauric acid (C12:0)
myristic acid (C14:0)
margaric acid (C17:0)
linolenic acid (C18:3)
arachidic acid (C20:0)
behenic acid (C22:0)

The latter fatty acids generally occurred at low percentages in the plasma samples.

The relative proportion of *palmitic acid* was generally 15.8–22.4% in the plasma lipids; prepartal changes in its percentage were of low significance ($P < 0.05$). Its concentration tended to rise from the beginning of the last prepartal month, and was at peak during calving and the subsequent month, when it increased significantly ($P < 0.001$) over the "dry" value, then returned to approximately the prepartal level (Fig. 1).

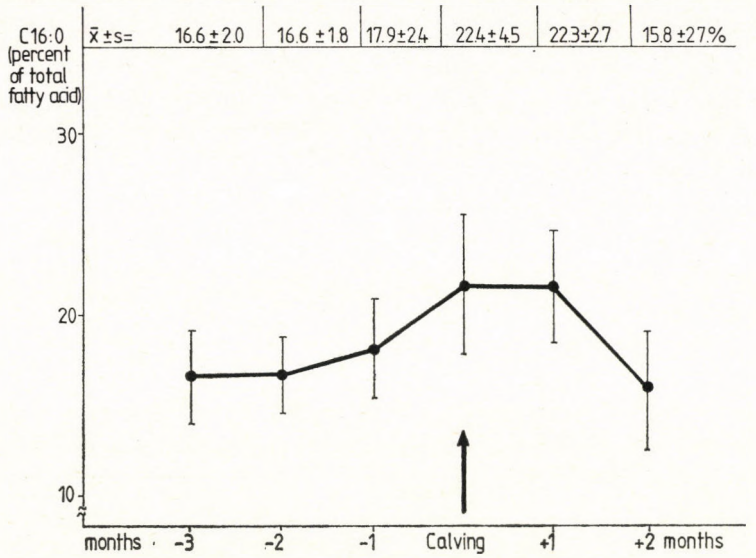

Fig. 1. Palmitic acid content of plasma lipids

The *palmitoleic acid* component, giving generally 2.1–4.1% of the plasma lipids, tended to decrease slightly before calving, increased considerably ($P < 0.01$) during calving and the subsequent month, and decreased again ($P < 0.005$) during the second month of lactation (Fig. 2).

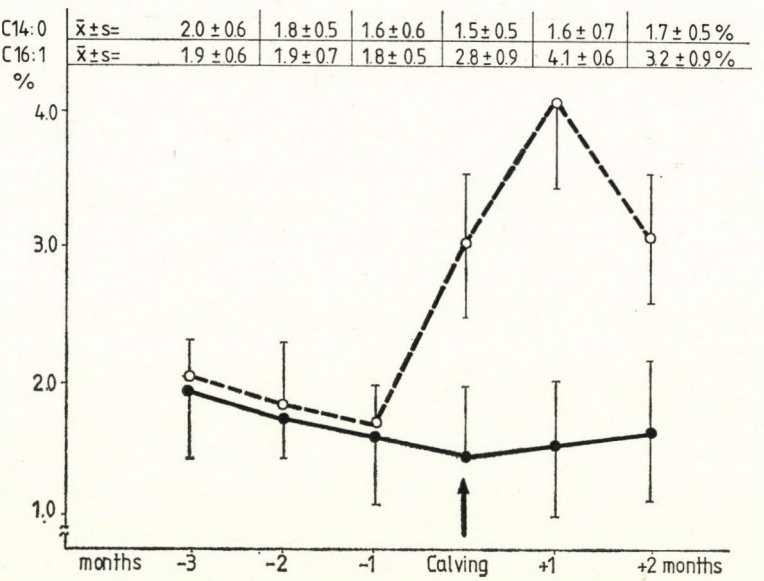

Fig. 2. Myristic and palmitoleic acid contents of plasma lipids. Solid line, C14:0, broken line C16:1

Stearic acid and *oleic acid* represented together 27–30% of the fatty acid components in plasma lipids. Their concentration changes having been opposite in the period studied, they gave a C18:0 + C18:1 maximum ($\bar{x}$ = = 30.4%) at calving, and a minimum ($\bar{x}$ = 26.8%) during the initial months of lactation. The plasma level of *stearic acid* was relatively high ($\bar{x}$ varied between 17.2 and 18.3%) and showed no appreciable ($P > 0.05$) fluctuation during the prepartal period, dropped to 60–70% of the prepartal level during calving, and remained low relative to the "dry" value for the first two months of lactation. Against this the *oleic acid* concentration increased almost twice over the prepartal value ($\bar{x}$ varied between 8.2 and 11.7%) during calving; postpartally, however, it decreased gradually ($P < 0.001$) (Fig. 3).

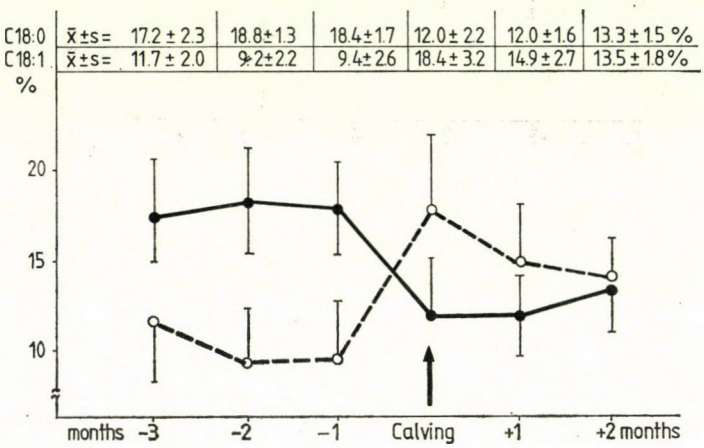

Fig. 3. Stearic and oleic acid contents of plasma lipids. Solid line, C18:0, broken line C18:1

Linoleic acid represents the largest component of plasma lipids, making up generally 36–44% of these. Its relative percentage fell to a minimum at calving, between pre- and postpartal peaks ($P < 0.01$). The *arachidonic acid* concentration curve ran approximately parallel with the linoleic acid curve and the changes were also significant ($P < 0.01$), but C20:4 represented only a minor component ($\bar{x}$ = 1.5–3.1%) of the plasma lipids (Fig. 4).

b) *Analysis of liver tissue*

The hepatic TL concentration was on the average 46.8±6.1 g/kg at 3 months before calving, essentially the same at 2 months, and slightly higher at 1 month before calving. The highest mean value, with considerable standard error ($\bar{x}$ = 76.6±21.0 g/kg) was found at calving ($P < 0.001$). Calving was followed by a decrease, especially during the 2nd postpartal month; the difference from the 2nd and 3rd prepartal months was not significant ($P > 0.05$) (Fig. 5).

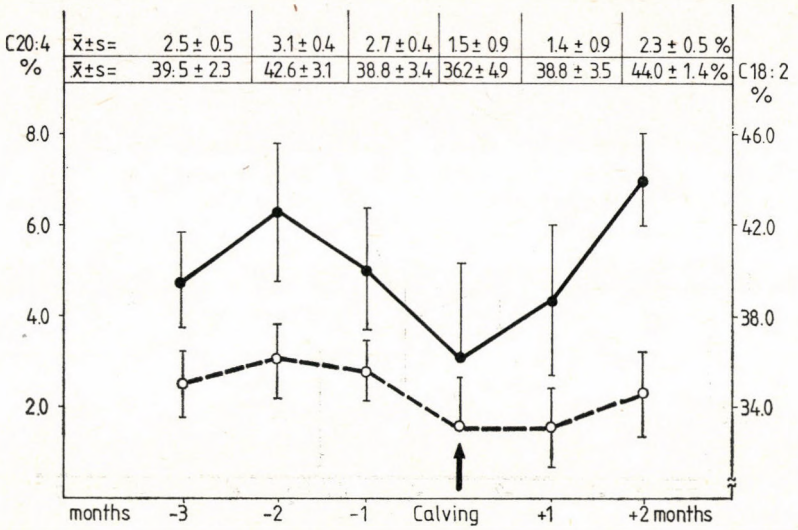

Fig. 4. Linoleic and arachidonic acid contents of plasma lipids. Solid line C18:2, broken line C20:4

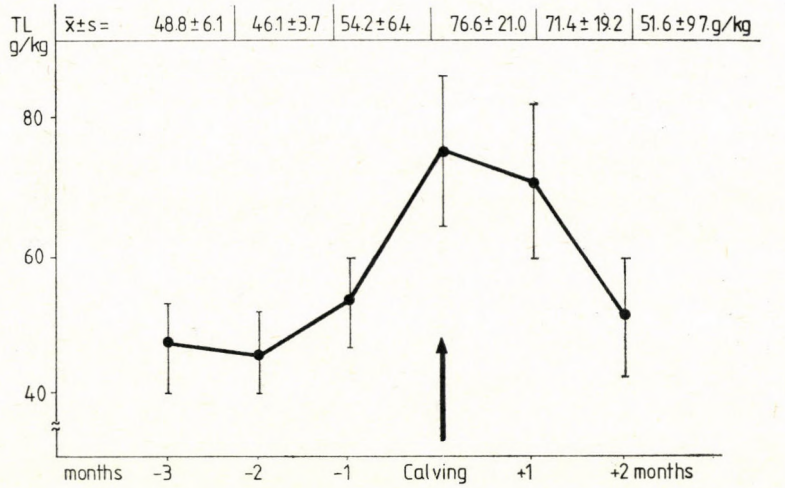

Fig. 5. Total lipid concentration (g/kg) in the liver

Percentual fatty acid composition of hepatic lipids. The hepatic lipids could be separated into the same 12 fatty acid components as the plasma lipids, and the same fatty acid components showed significant or non-significant fluctuations in the liver and plasma.

Palmitic acid, giving on the average 17–32% of the hepatic lipids, showed a gradual concentration decrease ($P < 0.05$) before calving, reached a peak

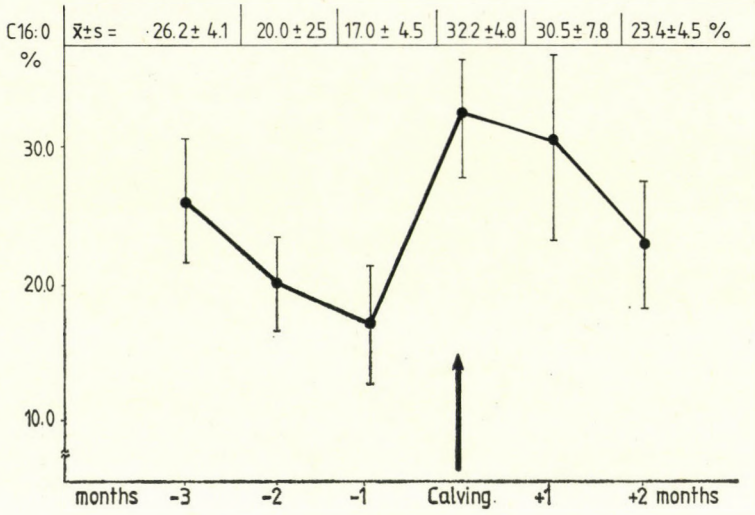

Fig. 6. Palmitic acid content of hepatic lipids

during it, then tended to decrease again ($P < 0.05$); the values found 3 months before and 2 months after calving were close to one another (Fig. 6).

Palmitoleic acid, representing 1.2–4.5% of the hepatic lipids, did not appreciably change during the prepartal period, having persisted below 2% throughout. It increased to more than double the prepartal value during calving ($P < 0.001$), and persisted above 4% during the first postpartal month. There was a gradual postpartal decrease ($P < 0.05$), but the C16:1 percentage was still about twofold the last prepartal value during the 2nd month of lactation (Fig. 7).

The curve for *myristic acid* is also shown in Fig. 7.

Fluctuations in the percentages of *stearic acid* and *oleic acid* showed in the hepatic lipids, just like in the plasma lipids, opposing trends. The mean percentage occurrence of C18:0 + C18:1 was assessed as 49–50%. This percentage is about twice greater than the one found in the blood plasma, thus C18:0 and C18:1 represent decisive fatty acid components in hepatic lipids, and the fluctuations of the percentage of C18:0 + C18:1 also are greater in the liver than in the blood. The contribution of C18:0 + C18:1 was the lowest ($\bar{x} = 47.2\%$) during calving, and showed opposing trends of increase towards the early prepartal and late postpartal sampling times.

The mean percentage for *stearic acid* was 37.5–39.5 before calving, and less than half of that value during calving ($P < 0.001$). It did not appreciably change for one month postpartum, and although it increased significantly ($P > 0.001$) over the parturient level at 2 months post partum, it was still significantly lower than the prepartal level.

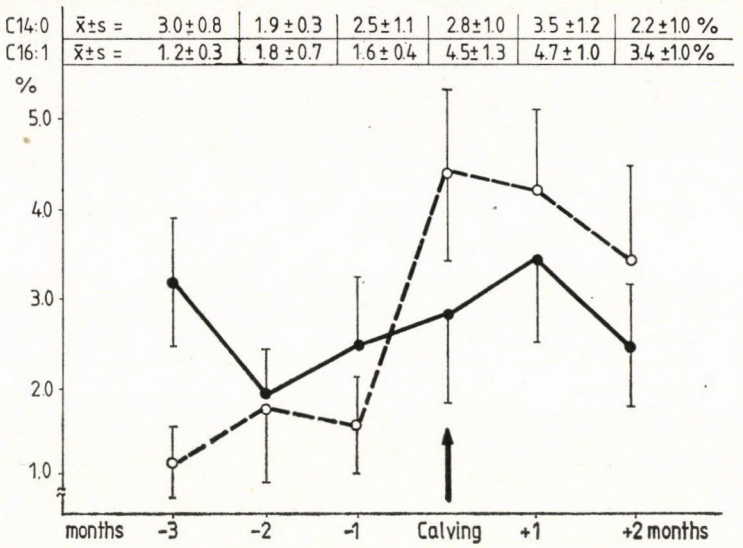

Fig. 7. Myristic and palmitoleic acid contents of hepatic lipids. Solid line C14:0, broken line C16:1

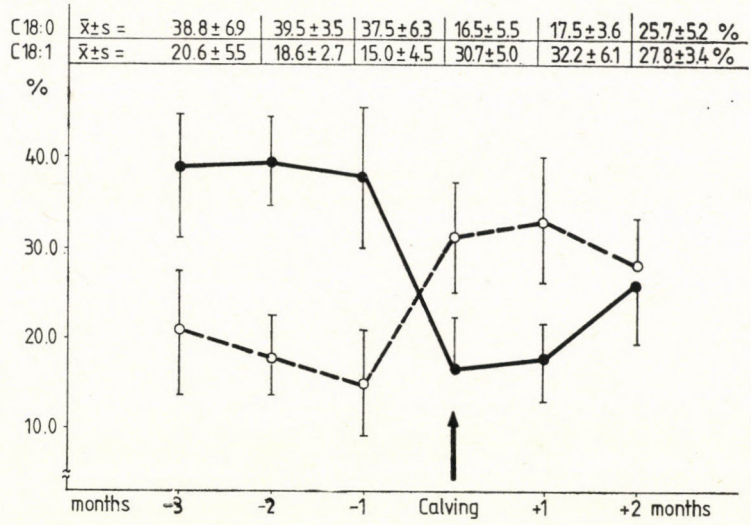

Fig. 8. Stearic and oleic acid contents of hepatic lipids. Solid line C18:0, broken line C18:1

Unlike stearic acid, the mean *oleic acid* content of the hepatic lipids rose from the prepartal value of 15.0–20.6% to 30.7 and 23.3% at calving and one month later, respectively. A slight decrease did, however, follow during the 2nd postpartal month ($P < 0.05$; Fig. 8).

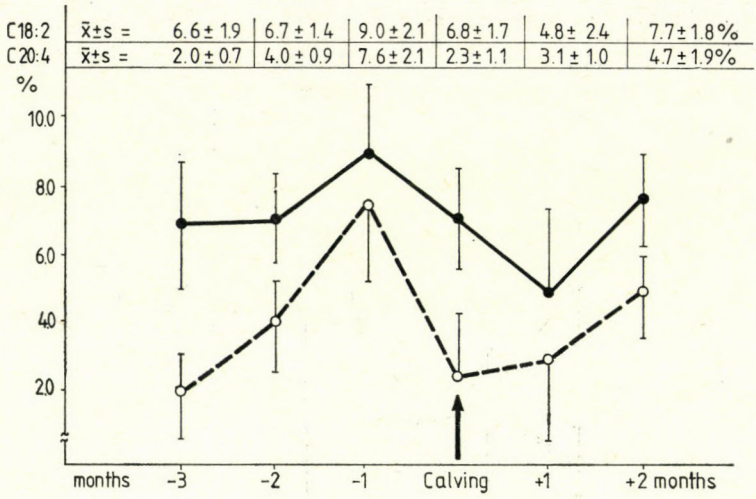

Fig. 9. Linoleic and arachidonic acid contents of hepatic lipids. Solid line C18:2, broken line C20:4

Changes in the percentages of *linoleic acid* and *arachidonic acid* showed an approximate parallelism also in the hepatic lipids. The contribution of linoleic acid to the TL was lower ($\bar{x}$ varied between 4.8 and 9.0%) in the liver than in the blood, thus it approximated that of arachidonic acid in the hepatic lipids. Hepatic C18:2 and C20:4 showed a concentration increase ($P < 0.05$) until 1 month before calving, and a considerable decrease ($P < 0.05$ and $P < 0.001$) at calving. The linoleic acid concentration fell to a minimum 1 month after parturition, the arachidonic acid concentration at parturition. Each minimum was followed by a significant ($P < 0.05$) increase (Fig. 9).

Discussion

In five-month model experiments we monitored certain parameters of lipid metabolism in properly managed and fed, healthy dairy cows, to obtain information on physiological fluctuations during the peripartal period. We determined the TL, TCh and FFA concentrations in the blood plasma, and the TL concentration in liver biopsies at one-month intervals from 3 months before till 2 months after parturition. We also identified the nature and estimated the percent participation of the fatty acid components in plasma and hepatic lipids in the preparturient, parturient and postparturient periods. Since plasma samples and liver biopsies were collected simultaneously and tested with the same methods, a comparison of the blood and liver parameters of lipid metabolism was also possible.

The plasma TL and TCh changes found by us followed essentially the pattern observed in similar studies by others but taking into consideration the exact time of parturition our investigations have thrown more light on certain aspects.

The plasma TL level of healthy dairy cows was on the average 35–36% lower at calving than during the dry period, but increased to more than double the calving level by the end of the 2nd month of lactation. We have found that the parturient healthy cow may show a TL drop as large as observed by Yamdagni and Schultz (1970) in ketotic cows.

The TCh level proved to be considerably lower during the immediate preparturient months and at calving than either in the earlier stage of pregnancy or during lactation. It follows that only significant decrease in plasma TCh (to less than 1.5 mmol/l) can be regarded as pathological in the period around calving.

The plasma FFA level increased abruptly, to about 3-fold of the "dry" value during calving, signifying a temporary, but considerable, energy deficit in the parturient period, by analogy of the changes observed by Brumby et al. (1975) in fasting cattle.

The obviously physiological nature of the dramatic rise of FFA during calving supports the implication (Adler et al., 1963; Kronfeld, 1965) that plasma FFA assays are of low value in the early diagnosis of peripartal disorders in bovine lipid metabolism.

In accordance with Pehrson's (1971) observation, we always found less than 5% FFA in the plasma lipids, but while the parturient concentration was on the average 4.5%, the pre- and postparturient levels were equally one-third or one-fourth of this value.

All cows developed a mild to moderate fatty liver at parturition, as signified by a more than 1.5-fold increase in the mean preparturient TL concentrations of 46.1 and 46.8 g/kg, assessed 3 and 2 months before calving, respectively. The individual variations in hepatocellular fat accumulation were great; the two highest values were 98.1 and 101.1 g/kg, the two lowest ones 62.4 and 65.9 g/kg. It should be noted in this context that other authors (Collins and Reid, 1980) found even greater TL than our higher limit in the liver of freshly-calved, healthy cows.

The accumulation of fat in the liver around calving observed by us caused neither clinical symptoms, nor other abnormalities detectable by the laboratory tests employed by us, and it was always transitory. The hepatic TL decreased to the last preparturient value during the second postpartal month, and the cows showed no sign of illness during further observation for a year.

It follows from the foregoing considerations that parturient changes in plasma and hepatic TL and certain main lipid components are similar to those

occurring in fasting cows. Increase in plasma FFA and hepatic TL, and decrease in plasma TL and TCh, take place equally under parturient and fasting conditions. These changes, which normalized soon after calving, can be regarded as physiological in the healthy parturient cow. The fatty acid composition of plasma and hepatic lipids was also followed up during the preparturient, parturient and postparturient periods.

Linoleic acid, palmitic acid, stearic acid and oleic acid-esters made up the bulk (85–90%) of the fatty acid components of plasma lipids.

The fatty acid components of hepatic lipids were the same as in the plasma, but their proportions were different. The hepatic lipids contained almost twice as much stearic and oleic acid, and more palmitic acid, than the plasma lipids; the latter three fatty acids made up 70–80% of hepatic TL. Of the other fatty acids, linoleic acid made up only one fifth to one eighth of the amount found in the plasma. Palmitoleic acid and arachidonic acid reached together a mean concentration of 4.5–7.5% in certain time periods.

The dissimilar percentage contributions of identical fatty acid components in plasma and liver are due to diversities in the quantitative relations of the main lipids. At parturition, triglycerides represent the bulk of the hepatic lipid, while the plasma triglyceride level is almost negligibly low. The triglycerides differ in fatty acid composition from the other lipids.

The fatty acid components of the plasma and hepatic lipids changed characteristically with pregnancy, calving and lactation.

The curves of stearic and oleic acid showed practically the same opposing course for plasma and liver. The sum C18:0 + C18:1 also was approximately identical in plasma and liver at all sampling times. The changes in palmitic acid concentration, too, were very similar in plasma and liver, and certain analogies were found between the fluctuations in the linoleic and arachidonic acid components as well.

It should be pointed out that the changes in the relative proportions of fatty acids were greater in the liver than in the plasma, and the standard errors of the mean values were also greater. It appears that the fatty acid complement of the blood lipids is more balanced than that of the liver.

The relative plasma levels of palmitic acid, oleic acid and palmitoleic acid showed an increase, whereas those of stearic, linoleic, arachidonic and myristic acid a decrease, in the parturient cow. The fluctuations observed in the fatty acid compositions of the plasma lipids were similar to those observed by Brumby et al. (1975) in fasting cows, and by Yamdagni and Schultz (1970) in ketotic cows.

Parturient changes in hepatic lipids involved the same fatty acid components as in the blood, but the quantitative relations of the concentration changes were dissimilar.

The present findings have indicated a connection between the param-

eters of lipid metabolism in plasma and liver. The concentration changes in plasma lipids were followed soon, and in many respects consistently, by similar alterations in liver lipids. It follows that plasma fatty acid levels inform us on the hepatic lipid status.

We assume that the physiological parameters of bovine lipid metabolism established in this study can serve as reference values for judging pathological alterations associated with disorders of lipid metabolism, such as fatty liver and ketosis. One should however be cautious in judging the pathological or non-pathological nature of certain changes. The healthy parturient cow, for example, may show dramatic changes in certain parameters of lipid metabolism relative to the preparturient "dry" values. While such changes are pathological in advanced lactation, they are fully physiological around calving.

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HISTOCHEMICAL STUDY OF PHOSPHATASES AND NONSPECIFIC ESTERASES IN THE HYPOPHYSIS CEREBRI OF GOAT *(CAPRA HIRCUS)* AND BUFFALO *(BUBALUS BUBALIS)*

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Cryostat sections of hypophyses of goat and buffalo were subjected to various histoenzymic tests for the demonstration of alkaline phosphatase, acid phosphatase, 5-nucleotidase, adenosine triphosphatase, glucose-6-phosphatase and nonspecific esterase.

The intensities of most enzyme activities varied considerably in different cell groups of the pars distalis of both species. The enzyme reactions were identical in the pars distalis of both species, except alkaline phosphatase activity, which was limited in the goat to the endothelium of capillaries, whereas, in the buffalo it was also evident intracellularly. Adenosine triphosphatase and 5-nucleotidase activities were lacking in the pars distalis of buffalo hypophysis.

All enzymes studied were present in varying amounts in the cells of the pars intermedia of both species, except adenosine triphosphatase and 5-nucleotidase, which could not be demonstrated even in this lobe of buffalo.

In the pars nervosa, both alkaline phosphatase and adenosine triphosphatase reactions were located in the endothelium in the goat, whereas only alkaline phosphatase was observed in the buffalo. The 5-nucleotidase enzyme could not be demonstrated in this lobe of either species. Acid phosphatase in pituicytes and nonspecific esterase along the nerve fibres presented the strongest reactivity.

Literature reveals studies on the histochemical localization of phosphatases and esterases in the hypophysis cerebri of monkey (Holmes, 1961), man (Fand, 1955), rabbit (Pearse, 1956), rat (Pearse, 1956; Samorajski, 1960; Pelietier and Novikof, 1972) and cat (Romieu et al., 1951). Of the ruminants only ox has been attempted to demonstrate the acid and alkaline phosphatases, adenosine triphosphatase and esterase, in the pars intermedia (Raftery, 1969). The present study was undertaken to demonstrate these enzymes histochemically in all the lobes of the hypophysis cerebri of goat and buffalo.

Materials and methods

The hypophysis cerebri of five female goats ranging in age between 6 months and 5 years, and a male buffalo of 3 or 4 years were sectioned at 10 μm thickness on cryostat microtome and were incubated for the following histoenzyme tests: (*i*) Alkaline phosphatase was determined with the azodye-

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coupling method after Burstone (1958*a*); (*ii*) acid phosphatase with the azodye-coupling method after Barka and Anderson (1963) modified from Burstone (1958*b*); (*iii*) 5-nucleotidase after Wachstein and Meisel (1956) and (*iv*) adenosine triphosphatase after Wachstein and Meisel (1956); (*v*) glucose-6-phosphatase after Wachstein and Meisel (1957); and (*vi*) nonspecific esterases after Barka and Anderson (1963).

Results and discussion

Different lobes of the hypophysis cerebri of goat as well as of buffalo presented varying intensities for almost all the histoenzyme tests performed. However, no glucose-6-phosphatase activity was recorded in any of the lobes in either species.

Table I

Relative intensities of enzymic activities in the parenchyma of hypophysis cerebri of goat and buffalo

Enzyme	Pars distalis		Pars intermedia		Pars nervosa	
	Goat	Buffalo	Goat	Buffalo	Goat	Buffalo
Alkaline phosphatase	(?)	+ to +++ (—)	(+ to ++)	(+)	(++++)	(++++)
Acid phosphatase	++++	+++	+	+	+++	+++
5-nucleotidase	+	—	+	—	—	—
Adenosine triphosphatase	+ to ++	—	±	—	(++ to +++)	—
Glucose-6-phosphatase	—	—	—	—	—	—
Nonspecific esterase	± to +++	++	±	±	++++	+++

++++, very strong; +++, strong; ++, moderate; +, weak: ±, very weak; ?, doubtful; —, negative reactivity.
Parentheses refer to reactions limited to the blood vascular wall.

The relative intensities for other enzymes studied in different lobes are summarized in Table I. Enzyme activities are discussed individually in the following.

Alkaline phosphatase (AKP)

Alkaline phosphatase activity was rare or absent in the cells of pars distalis adenohypophysis of the goat. Even the blood vessels were negative, except for rare granules in sinusoids. Samorajski (1960) also reported negative AKP activity in the cells as well as blood vessels of this lobe in the rat. However, he recorded the presence of nuclear staining and positive granules in pituitary

"β" cells, using Gömöri's cobalt method. The present authors are of the view that the possibility of the presence of intracellular AKP activity in the pars distalis cannot be ignored because in buffalo (Fig. 1) a varying (very weak to strong) degree of AKP activity was noticed in these cells. Such variation could very likely be attributed to the status of the cells and of the organs as a whole.

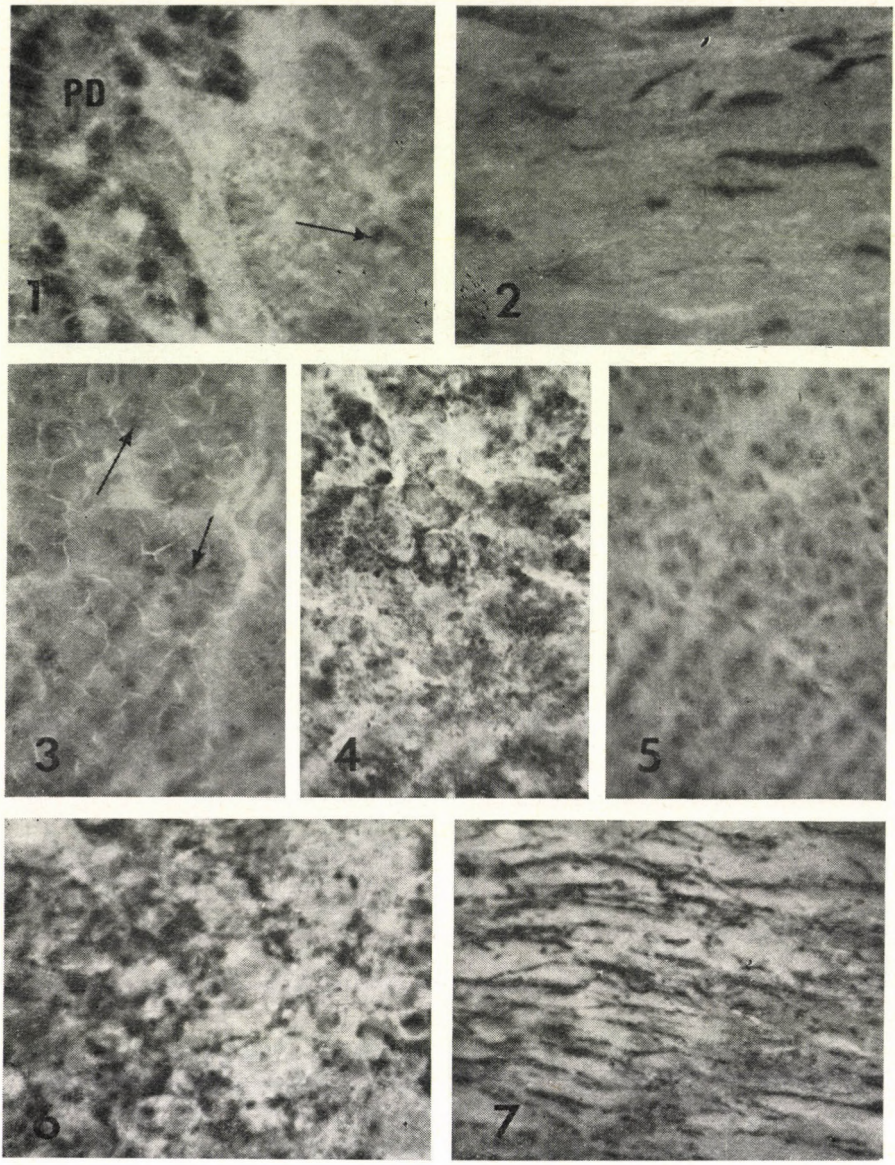
In the pituitary of the rat, blood vessels and their associated nerve fibres were intensely stained for AKP in the pars nervosa and comparatively weakly in the pars intermedia. The reaction was negative in the pars anterior, except for blood vessels at the periphery of the glands (Samorajski, 1960). No AKP activity has been reported in the pars intermedia of cat (Romieu et al., 1951). In the pars intermedia and the pars nervosa of goat (Fig. 2) as well as buffalo, strong AKP activity was present on the wall of blood vessels, weaker activity in the vessels of the pars intermedia. Thus, the present observations are more or less in agreement with those on monkey (Holmes, 1961), rat (Samorajski, 1960) and ox (Raftery, 1969).

These observations are suggestive of the role of AKP in dephosphorylation necessary for absorption and transport through the blood vessels in the pars intermedia and pars nervosa of goat, buffalo, rat, monkey and ox. But this function does not appear to be associated with AKP in blood vessels of the pars distalis of all these species except the buffalo. Another factor worthy of consideration is that fenestrated capillaries rarely exhibit AKP activity, and those of the pars distalis, being fenestrated and attenuated, their AKP activity may be below the threshold level of activity. Moreover, in the buffalo at least the presence of AKP activity in the cells of pars distalis is suggestive of its possible role in the control of intracellular metabolism by dephosphorylation, in the synthesis of some esterase, and probably also in the exchange of ions across the plasma membrane. However, the functions may be dependent on the functional status of various cellular components of the organ, and may be species-specific.

Acid phosphatase (ACP)

Strong ACP activity was seen in the majority of the cells of the pars distalis of the goat hypophysis (Fig. 3), concentrated chiefly near the nuclei and being indicative of lysosomal ACP. Certain sparsely-occurring undifferentiated cells were intensely reactive. These latter cells are most probably identical to the cells described by Barka et al. (1961) as ACP-reactive reticuloendothelial cells of the hypophysis cerebri. Also in the buffalo, an identical pattern of reaction was observed.

ACP activity was weaker in the pars intermedia cells of the goat and buffalo. It was not found in the nuclei, and this negative finding disagrees



Figs 1—7. 1. Alkaline phosphatase activity in pars distalis cells (PD) and blood vessels of pars intermedia (arrow) of buffalo, ×200. *2.* Alkaline phosphatase activity in blood vessels of pars nervosa of goat, ×200. *3.* Acid phosphatase activity (arrows) in the pars distalis cells of goat, ×450. *4.* ATPase activity, stronger in the periphery of pars distalis cells of goat, ×450. *5.* 5-nucleotidase activity in form of granules in the cells of pars distalis of goat, ×450. *6.* Nonspecific esterase activity in the pars distalis of goat, ×200. 7. Nonspecific esterase activity in the pars nervosa of goat, ×100

with Raftery's (1969) report. We attribute the disagreement to technical differences.

In the pars nervosa of the goat hypophysis, the ACP activity of pituicytes chiefly appeared to be lysosomal. A much stronger activity at certain sites, probably in the nerve terminals and Herring bodies was also discernible, which can very well be correlated with the presence of strong ACP activity reported in the paraventricular and supraoptic nuclei of the hypothalamus (Eranko, 1951; Samorajski, 1960; Sloper, 1955). The ACP activity was noticed to be comparatively strong in older subjects.

Adenosine triphosphatase (ATPase)

ATPase activity varying in intensity from very weak to moderate was observed in different cells of the pars distalis of the goat hypophysis (Fig. 4). The activity was very weak in the cells of the pars intermedia. In the buffalo, ATPase could not be demonstrated in any of the hypophyseal lobes. In the ox, ATPase activity has been reported to be present in the large vessels and capillaries of pars intermedia (Raftery, 1969). This was not found in the goat. In the pars nervosa, weak to moderate ATPase reactivity was seen on the wall of capillaries and large blood vessels. This indicates that the energy requirement for the transport mechanism of secretory products across the capillaries of pars nervosa is probably furnished by the breakdown of ATP as well as by phosphorylation, by the latter process only in the pars intermedia and by some mechanism other than either of the two in the pars distalis of the goat hypophysis.

5-Nucleotidase (5-NT)

5-NT reactivity was observed within the cells of pars distalis and pars intermedia in form of one to three or more coarse granular precipitates (Fig. 5). No activity could be observed in the pars nervosa. In the pituitary of the buffalo no 5-NT activity was demonstrated in any part. Amongst all tissues, strongest 5-NT activity has been reported in the human posterior pituitary after an incubation period of 3 h (Pearse and Reis, 1952). The present observations are not comparable to the latter study because the incubation period was only 90 min.

Nonspecific esterases (Ease)

Ease, as demonstrable by naphthyl acetate (hexazonium pararosaniline technique), was observed to be very variable, being strong in some and very weak in other cells of the pars distalis (Fig. 6). Fand (1955) demonstrated Ease

in the basophils of the human pituitary. In the present study, simultaneous identification of various cell types was not undertaken.

The cells of pars intermedia of goat and buffalo showed a very weak but uniform Ease activity in all the cells. The presence of Ease has also been reported in this lobe of rabbit and rat (Pearse, 1956). In ox, Ease activity was intense in some cells but weak in others, probably due to the two types of cells in pars intermedia as has been described by Raftery (1969). But this does not appear to be the case in goat and buffalo. The pars intermedia being the only lobe of adenohypophysis where nerve fibres are present in association with cells, esterase activity observed in this lobe of different species can very well be appreciated.

In the pars nervosa of the goat hypophysis (Fig. 7), very strong Ease activity was noticed, chiefly along the nerve fibres and in the Herring bodies. Similarly localized, but weaker, activity was found in the buffalo. The aliesterase activity has also been shown to be strong in paraventricular and supraoptic nuclei (Adams et al., 1960; Pepier and Pearse, 1957; Sloper, 1955). Raftery (1969) recorded a stronger Ease activity in the pars intermedia than in the pars nervosa of the ox. However, in goats much more intense Ease activity is in the pars nervosa than in the pars intermedia of this species.

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EXPERIMENTAL STUDY OF GENERALIZED CANINE TOXOPLASMOSIS

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Forty-eight 1–3 months old dogs of unknown serological background were infected experimentally with *Toxoplasma gondii* cysts or oocysts. Part of the dogs was subjected to immunodepressive treatment with the synthetic corticosteroid betamethasone (Betsolan inj., Glaxo) (Table I). One third of the dogs infected with cysts or oocysts and treated with betamethasone developed a lethal generalized toxoplasmosis by 2–3 weeks after experimental infection. In part of the cases, a non-generalized toxoplasmosis developed, and no infection could be established in about half of the experimental animals. In the group without immunodepressive treatment only a single animal developed generalized toxoplasmosis, and non-generalized cases were considerably less frequent than in the group of dogs given the immunodepressant (Table II). The experimental observations thus support the implication that generalized toxoplasmosis develops in dogs primarily as a sequel to the suppression of immune responsiveness by a disease or some other factor.

Dogs died in naturally acquired generalized toxoplasmosis usually developed the condition in association with canine distemper (Yakimoff and Kohl-Yakimoff, 1911; Campbell et al., 1955; Hartley, 1956; Møller and Nielsen, 1964; Capen and Cole, 1966; Dobos-Kovács and Kardeván, 1976; Girád, 1979), less often in association with other primary diseases, such as chronic lead poisoning (Hartley, 1956), lymphoid leucosis (Alcaino et al., 1964), etc. It has been suggested that depression of immunological responsiveness by the primary disease predisposes the dogs for generalized toxoplasmosis. In certain cases, however, evidence of any other pathological condition was lacking in dogs diseased in generalized toxoplasmosis (Hartley, 1956; Capen and Cole, 1966; Dobos-Kovács and Kardeván, 1976; Girád, 1979).

We investigated the pathogenesis of generalized canine toxoplasmosis in dogs experimentally infected with *Toxoplasma gondii* cysts or oocysts by oral route, under simultaneous induction of chemical immunodepression.

Materials and methods

A total of 48 dogs of unknown serological background, 1–3 months old at the beginning of the experiment, were used. The dogs were infected orally with *Toxoplasma gondii* cysts or oocysts, in five successive experiments (I–V), performed either on own-bred (group I and II) or purchased animals originating from two litters each (Table I).

The *Toxoplasma gondii* strain used for experimental infection was isolated from the organs (liver, lung, brain) of a dog spontaneously died in generalized toxoplasmosis supervening on canine distemper. Isolation was effected by oral administration of the pooled organ suspension to albino mice and the isolate was maintained by serial passage.

Toxoplasma gondii cysts were obtained for the experimental infection of dogs as follows: the brain suspension of infected mice was checked for presence of cysts by microscopic examination, and was administered orally to albino mice. The brain suspensions of the recipient mice were used three weeks later for the experimental infection of dogs, each receiving about 800 cysts.

Toxoplasma gondii oocysts were isolated from the faeces of experimentally infected young cats, and were used for the experimental infection of dogs after sporulation. Each dog received about 50,000 oocysts.

Immunodepression was induced in part of the dogs (25 animals) by intramuscular treatment with a synthetic corticosteroid (betamethasone, Betsolan inj., Glaxo Laboratories, U.K.) at 0.8 mg/kg bwt dose level on the day preceding experimental infection and every other day after it (experimental group). The remaining 23 dogs infected with *T. gondii* received no immunodepressive treatment (control group).

The dogs which died due to the infection and the survivors exterminated after the conclusion of the experiment (4 weeks p.i.) were autopsied. During post mortem examination organ samples were secured for histological examination from the small intestine (several segments), mesenteric lymph node, liver, lung, pancreas, adrenals, spleen, thymus, heart, diaphragmatic and skeletal muscles, and brain. The samples were fixed in 8% (2.66 mol/l) formalin and were embedded in paraffin. The paraffin sections were stained with haemalaun and eosin; some sections from the lung were stained with Endes's trichrome stain for fibrin detection.

Liver and brain suspensions from each dog were used for oral infection of albino mice. Three weeks later the mice were killed and their brains were examined by native microscopic technique for the presence of *Toxoplasma* cysts (mouse inoculation test).

Some 21 and 20 dogs of the experimental and control groups, respectively, were weighed and the absolute and relative weights of the lymphoid organs (thymus and spleen) were recorded (Table III).

Results

I. *Generalized toxoplasmosis*

In the group given immunodepressive treatment, 6 of 16 cyst-infected dogs died in generalized toxoplasmosis 8, 9, 14, 16, 16 and 20 days p.i., and 2 of 9 oocyst-infected dogs 15 and 17 days p.i., respectively. In the group not

treated with betamethasone, one of 6 dogs infected with oocysts died also in generalized toxoplasmosis 17 days p.i. (Table I).

Table I

Schedule and results of the experiments

Experimental series No.	n	No. of dogs infected/died in generalized toxoplasmosis			
		Betamethasone		No betamethasone	
		Toxoplasma gondii cysts	*Toxoplasma gondii* oocysts	*Toxoplasma gondii* cysts	*Toxoplasma gondii* oocysts
I	9	5/3	—	4/0	—
II	10	5/3	—	5/0	—
III	8	2/0	2/1	2/0	2/0
IV	11	4/0	3/0	4/0	—
V	10	—	4/1	2/0	4/1
Total	48	16/6	9/2	17/0	6/1
		25/8		23/1	

Post mortem findings

The *spleen* of the dogs which died in generalized toxoplasmosis was usually normal, less often slightly swollen. The liver was in most cases enlarged, yellowish-brown or brownish red; in every case, few to many greyish-white or greyish-red foci, the size of a pin-prick, pin-head, or, infrequently, a millet seed, could be seen in the liver parenchyma. The foci were not infrequently surrounded by a 1 mm wide, greyish-white halo (Fig. 1). The parenchymal structure was always indistinct on the cut surface, and the consistence of the tissue was friable. In no case was a gastro-intestinal change found. The *mesenteric lymph nodes* were in most cases enlarged, and their cut surface was juicy. Circumscribed or diffuse dry regions were frequently found in the lymphonodular parenchyma.

In the *lung* there were either numerous small (pin-prick) or few large (size of a millet seed) greyish-white compact foci. In part of the cases the focal lung lesions were grossly inapparent, and seen only in histological sections.

Other organs showed no alteration at post mortem examination.

Histological findings

The focal *liver* lesions represented essentially four histological types. In part of the cases loss of parenchymal structure and hepatocellular necrosis

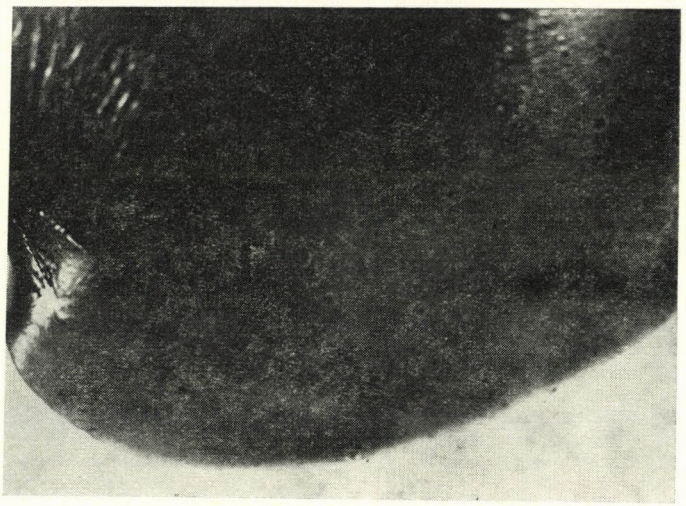

Fig. 1. Necrotic-inflammatory foci, pin-prick to the size of a millet seed, in the liver tissue. Many necrotic-haemorrhagic foci, surrounded by a pale halo, are interspersed between the necrotic-inflammatory lesions. Gross appearance, slightly magnified

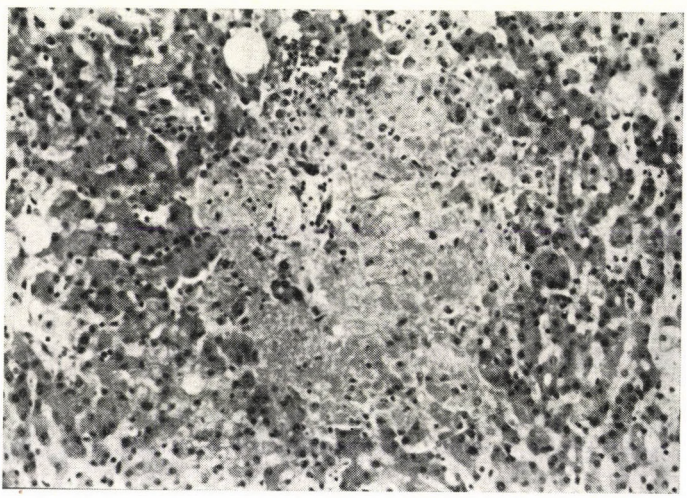

Fig. 2. Low-power view of a pure necrotic focus in the liver parenchyma. H. and E. (×160)

could be seen in the area of focal change, without surrounding inflammatory reaction (pure necrotic foci) (Fig. 2). Around most necrotic foci tachyzoites established themselves in extra- and intracellular localizations (Fig. 3). In other cases there were many free erythrocytes in the centre of the hepatocellular focal necrosis (necrotic-haemorrhagic foci); extracellular solitary tachyzoites, and/or intracellular aggregations of these also occurred in the surroundings of such lesions. Foci of the third type consisted of a centrally placed

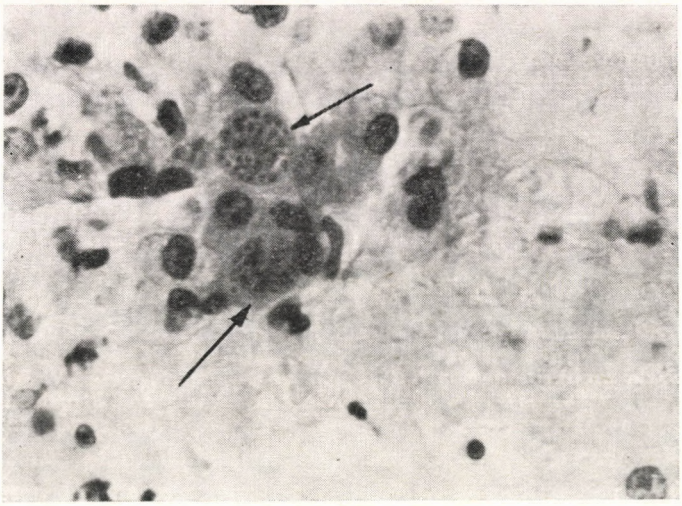

Fig. 3. Intracellular groups of tachyzoites (↗) in liver cells adjoining the necrotic focus. H. and E. (×1000)

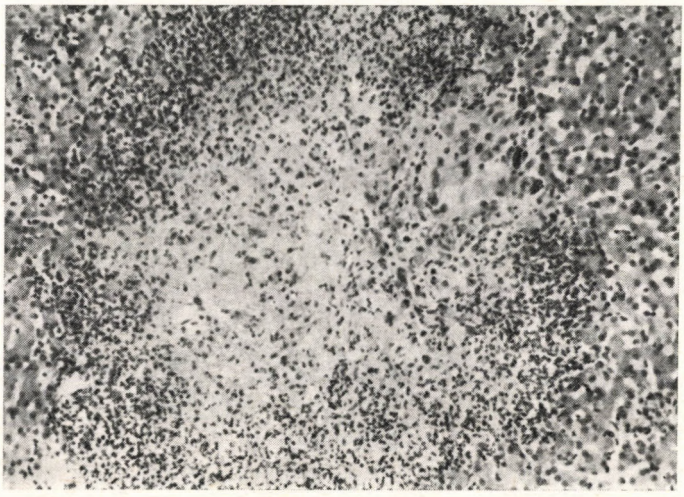

Fig. 4. Necrotic-proliferative focus in the liver parenchyma. H. and E. (×160)

pure necrotic area, surrounded by a halo of infiltrating cells, for the most part reticulum cells (necrotic-proliferative focus) (Fig. 4). The fourth histological type was represented by foci formed exclusively of proliferating reticulum cells (pure proliferative focus) (Fig. 5). Developmental stages of *T. gondii* appeared hardly, if at all, near to necrotic-proliferative and pure proliferative foci. Foci of all four types occurred mainly in the periphery of liver lobules.

In most cases, apart from necrotic-haemorrhagic foci only a single other type of focal lesion (pure necrotic, necrotic-proliferative or pure prolifer-

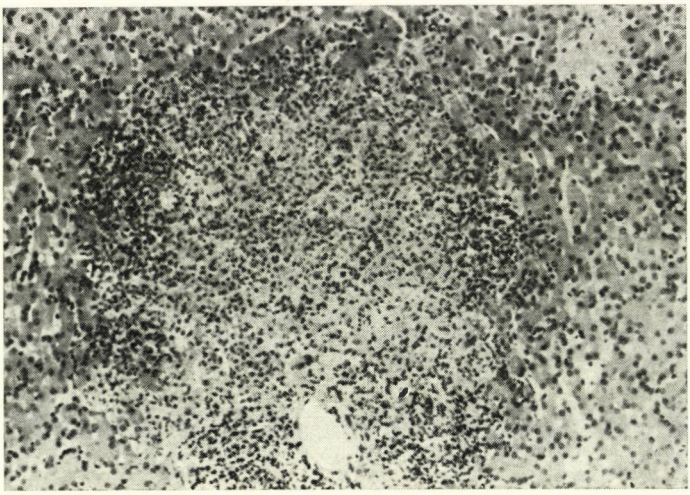

Fig. 5. Pure proliferative focus in the liver parenchyma. H. and E. (×160)

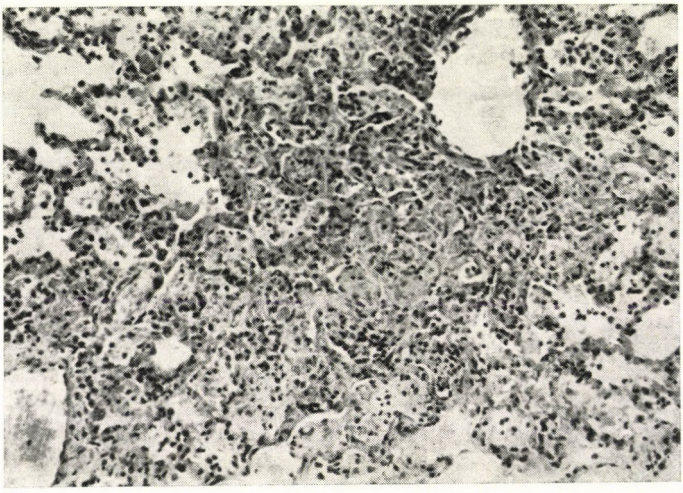

Fig. 6. Low-power view of an inflammatory-necrotic focus in the lung tissue. The alveola lumina are filled by a fibrinous exudation admixing with minor to major amounts of nuclea detritus. H. and E. (×60)

ative) was represented in the liver of a host, but in a few cases all four types were present simultaneously. It should be noted in this context that a single dog with generalized toxoplasmosis, of the group not given immunodepressive treatment, had no necrotic-haemorrbagic foci in the liver but did have foci of all three other types.

In the *lung*, microscopic lesions involving few to many alveoli could be seen near to small vessels. The alveoli were enlarged, and a homogeneously staining, infrequently stratified exudation was present in their lumina. The

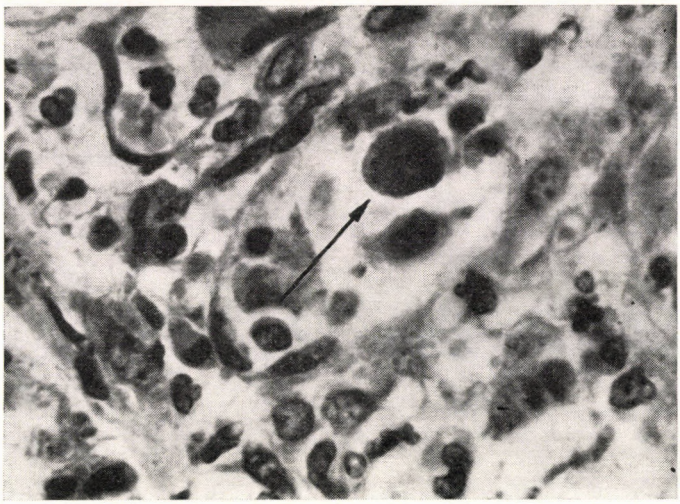

Fig. 7. Group of tachyzoites (↗) in an alveolar epithelial cell near to an inflammatory-necrotic pulmonary focus. H. and E. (×1000)

homogeneous-appearing exudate contained a considerable amount of fibrin, a varying amount of cell debris, a few desquamated alveolar epithelial cells, 1–2 neutrophilic granulocytes and solitary tachyzoites (Fig. 6). In several cases groups of tachyzoites could be seen inside alveolar epithelial cells (Fig. 7). The above pulmonary lesions were present in all lungs except that of the dog which died 8 days after experimental infection.

Cerebral lesions caused by toxoplasma stages were found in the *brain* of all dogs except those died 8 and 9 days after infection. Most lesions were identified as circumscribed microscopic necroses, and groups of tachyzoites could be seen in many adjoining nerve and glia cells. In other cases intracellular aggregations of tachyzoites occurred in perivascular localizations, in association with a focal proliferation of glia cells (Fig. 8). Infrequently the cells adjoining glia cells carrying tachyzoites or bradyzoites were not involved by either necrotic or inflammatory change (Fig. 9). Occasionally no *Toxoplasma* stages could be seen inside and in the surroundings of circumscribed glia cell proliferations. The focal necrotic-inflammatory lesions caused by the developmental stages occurred for the most part in the cerebral cortex, less often in the striated body, Varol's bridge or medulla oblongata.

The soft meninges of the dogs which died 8 and 9 days after infection were thickened by serous and cellular (neutrophilic) infiltration. Bacterial "clouds" were also present in the meningeal region involved by acute purulent meningitis. One of the above dogs also had bacterial "clouds" in the lumina of part of the renal and hepatic interstitial small vessels. Around the latter, infiltrating pus cells appeared in the hepatic interstitial tissue.

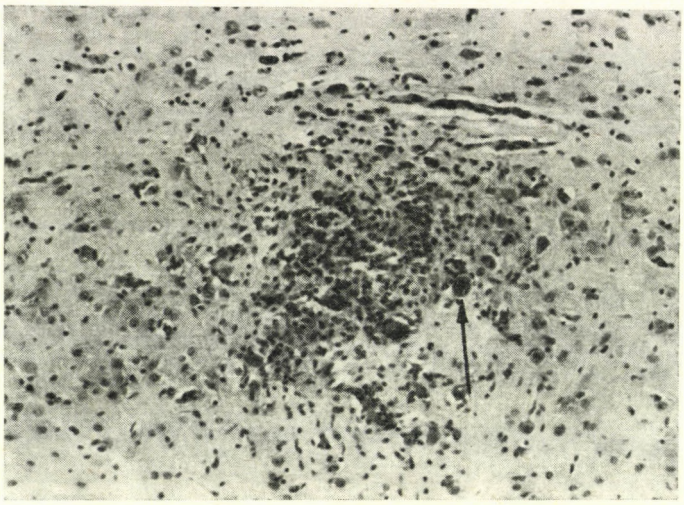

Fig. 8. Perivascular focal glia cell proliferation. A cyst near to the focus (↗)

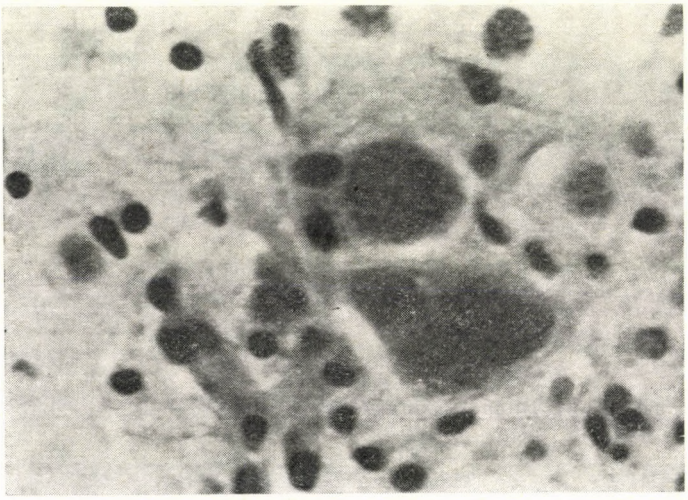

Fig. 9. Groups of bradyzoites (initial stages of cyst formation) in the cytoplasm of nerve cells. There is no inflammatory cellular reaction around the cysts. H. and E. (×1000)

In several cases groups of tachyzoites localized in *myocardial* cells, which were surrounded by a circumscribed histio-lymphocytic infiltration.

In the *spleen*, focal necrosis of some Malpighian corpuscles, and presence of groups of tachyzoites in the surrounding reticulum cells, was infrequently seen.

In about half of the cases, necrosis of a few follicular cells or of entire follicles was observed in the cortical tissue of the *mesenteric lymph nodes*; occasionally extensive lymphonodular regions were involved by necrosis.

Near to the necrotic areas, a few solitary reticulum cells enclosed groups of tachyzoites.

Infrequently the intracellular occurrence of tachyzoite groups, accompanied by solitary cell necroses, was observed in some *lymph follicles of the small intestine.*

Mouse inoculation test

This was always positive in albino mice infected orally with liver and brain suspension from dogs died in generalized toxoplasmosis.

II. *Non-generalized toxoplasmosis*

Post mortem findings

No gross lesions indicative of toxoplasmosis were found in the carcases of dogs exterminated four weeks after experimental infection.

Histological findings

Three cyst-infected and three oocyst-infected dogs of the group given immunodepressive treatment, and 2 and 1 cyst- and oocyst-infected dogs, respectively, of the group not given betamethasone, showed microscopic lesions indicative of toxoplasmosis in different organs varying between hosts (Table II).

Table II

Results of the infection experiments

	No. of dogs					
	Betamethasone			No betamethasone		
	T. gondii cysts	*T. gondii* oocysts	Total	*T. gondii* cysts	*T. gondii* oocysts	Total
Generalized toxoplasmosis	6	2	8	—	1	1
Non-generalized toxoplasmosis	3	3	6	2	1	3
No toxoplasmosis developed	7	4	11	15	4	19
Total number of dogs	16	9	25	17	6	23

Of the six dogs given betamethasone, five harboured intracellular groups of tachyzoites in the heart muscle, and one harboured them in the diaphragmatic muscle. The tachyzoite-carrying cells were not surrounded by cellular reaction in the great majority of the cases, and only by a very mild histiolymphocytic infiltration in the remaining few cases. Three of the above six

dogs also harboured intracellular aggregations of tachyzoites not accompanied by cellular reaction in brain cells, as well as developing cysts inside cerebral glia and nerve cells.

Of the three dogs exhibiting microscopic lesions in the group not given immunodepressive treatment, two showed the changes observed in the betamethasone-treated dogs in the brain, one in the heart muscle.

No microscopic lesions attributable to *Toxoplasma* infection were found in the other organs (liver, lung, etc.) of the experimental and control animals.

Mouse inoculation test

Contrary to our expectations, oral infection of albino mice with liver and brain suspensions from the above dogs proved negative.

III. *Negative results of experimental infection*

The other dogs exterminated after the conclusion of the experiment, i.e. 11 and 19 of 25 and 23 animals, respectively, of the groups treated and not treated with betamethasone, showed neither gross nor microscopic indications of infection with *T. gondii* (Table II).

Mouse inoculation test

With liver and brain suspensions from these dogs, the test showed negative results.

The immunosuppressive treatment accounted for a significant decrease in the relative mass of the thymus and adrenals (Table III).

Table III

Mean body weight and relative organ weights ($\bar{x} \pm SE$) of dogs treated ($n = 21$) and not treated ($n = 20$) with betamethasone

	Treated	Not treated	p*
Body weight (g)	2549 ± 309	2695 ± 325	>0.05
Thymus (mg/100 g bwt)	28.3 ± 4.3	110.4 ± 13.7	<0.001
Spleen (mg/100 g bwt)	258.8 ± 27.1	255.9 ± 19.7	>0.05
Adrenals (mg/100 g bwt)	16.0 ± 1.0	23.3 ± 2.2	<0.05

* assessed by Student's *t* test

Discussion

The pathological criterion of generalized toxoplasmosis is the simultaneous involvement of several organs by distinct gross and/or microscopic inflammatory-necrotic focal lesions elicited by developmental stages of *T. gondii*.

In the present experiments generalized toxoplasmosis could be equally induced with *T. gondii* cysts or oocysts in dogs given betamethasone (Betsolan sol. inj.) for chemical immunosuppression. About one third of the experimental animals died in generalized toxoplasmosis 2–3 weeks after infection. In a few cases death occurred earlier, on days 8 and 9 p.i. These dogs developed, apart from generalized toxoplasmosis, bacteriaemia or purulent bacterial leptomeningitis owing to immunosuppression, and the bacterial complication accounted for early death.

In groups I and II, which represented litters reared in our Department in conditions exclusive of *Toxoplasma* infection, the subgroups given immunosuppressive treatment showed a greater incidence of generalized toxoplasmosis than similarly treated dog subgroups of unknown history, purchased for the purpose of the experiment.

A single control animal, infected with oocysts, developed generalized toxoplasmosis. In that particular case there was no obvious reason for the presumably reduced immune responsiveness of the host.

Apart from minor individual variations, no appreciable difference was found between the nature and morphology of the gross and microscopic lesions by cyst-infected and oocyst-infected hosts showing generalized toxoplasmosis.

The type of the lesions found in the liver, lung, brain and other organs of dogs given immunodepressive treatment before and during experimental infection was essentially similar to that observed by us (Dobos-Kovács and Kardeván, 1976) and others in naturally occurring (Campbell et al., 1955; Hartley, 1956; Møller and Nielsen, 1964; Capen and Cole, 1966; Girád, 1979) and experimentally induced cases (Vallée et al., 1961; Capen and Cole, 1966) of generalized toxoplasmosis.

The necrotic-inflammatory foci were more frequent in the liver of the experimentally infected dogs than in distemper-associated natural cases of toxoplasmosis, and also were grossly more conspicuous. Haemorrhagic-necrotic foci were not seen in natural cases, nor in experimentally induced toxoplasmosis without immunodepressive treatment, having been present exclusively in conditions of artificial immunodepression. The reason of this might perhaps be some specific action of the applied chemical immunodepressant (betamethasone).

The lung lesions were considerably less distinct and severe in experimental cases with immunosuppression than in natural cases of generalized toxoplas-

mosis supervening on canine distemper. Presumably a local disturbance of the pulmonary immune mechanisms, particular to the latter disease, may also account for the serious lung involvement. The nature of the pulmonary lesions (serous-fibrinous necrotic-inflammatory focal change) was essentially similar in the natural and artificially induced cases of toxoplasmosis, as suggested also by others (Capen and Cole, 1966).

Microscopic cerebral necroses (encephalomalacia) were less frequent in experimental toxoplasmosis than in natural disease supervening on canine distemper, but for the time being even a hypothetical explanation is lacking to this end.

Part of the dogs (about one fourth and one seventh to one eighth in the experimental and control subgroups, respectively) showed no generalization of toxoplasmosis in the pathological sense. In these cases developmental stages of *T. gondii* were demonstrable in certain organs (brain, heart muscle, diaphragmatic muscle), as a rule without a surrounding cellular reaction. These particular cases satisfied the parasitological criteria of generalized infection, but not the pathological criteria which foresee the simultaneous presence of characteristic necrotic-inflammatory foci in several organs.

As to the prevalence of non-generalized toxoplasmosis, it was greater in both cyst-infected and oocyst-infected dogs of the group given immunosuppressive treatment than in that of control. This seems to suggest that different degrees of immunosuppression may promote the development and multiplication of *T. gondii*.

In the major part of the cases (more than half and more than three quarters of the experimental and control dogs, respectively) no generalized toxoplasmosis demonstrable with the applied methods developed either by cyst-infected or oocyst-infected hosts. Apart from individual variations in susceptibility, immunity stimulated by an earlier *Toxoplasma* infection during the unknown history of the hosts may have been responsible for insusceptibility in such a considerable part of the cases.

The observations of other authors and our own experience equally suggest that, apart from infections by highly pathogenic *Toxoplasma* strains, dogs develop generalized toxoplasmosis primarily as a sequel to reduced immune responsiveness. In this light the nature of the factor responsible for seems to be indifferent, be it canine distemper, chronic lead poisoning, lymphoid leucosis or a chemical immunodepressant, or even some idiopathic factor.

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TERATOGENICITY OF METHYLPARATHION 18 WP AND WOFATOX 50 EC IN JAPANESE QUAIL AND PHEASANT EMBRYOS, WITH PARTICULAR REFERENCE TO OSTEAL AND MUSCULAR SYSTEMS

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Insecticides Methylparathion 18 WP and Wofatox 50 EC, formulated as waterable powder and emulsion concentrate, respectively, were tested for teratogenicity in Japanese quail and pheasant embryos, with particular reference to osteal and muscular systems.

Both pesticides were diluted in water to three various concentration levels and the dilutions were injected into the air cell in a volume of 0.05 ml/quail egg and 0.1 ml/pheasant egg.

The concentrations were 0.05, 0.5, and 5.0% for Methylparathion 18 WP and 0.02, 0.2, and 2.0% for Wofatox 50 EC. Quail eggs were treated on day 9, and pheasant eggs on day 12, of incubation. The results were evaluated on days 14 and 23 of incubation, respectively.

The applied test methods failed to demonstrate skeletal lesions in quail and pheasant embryos with evident lordoscoliosis (the most frequent deformation seen). The gross and microscopic appearance of the cervical vertebrae was usually normal. High doses of Wofatox 50 EC produced, however, atrophic and, occasionally, hypoplasic changes in the cervical muscle of the embryos. The authors call attention to the possibility that organophosphates for inhibition of embryonic innervation may be a contributive factor predisposing to lordoscoliosis. This state may secondarily induce atrophic changes of the cervical muscle and an abnormal posture of the neck.

It is likely that the tested pesticides are non-hazardous to the developing avian embryo under the conditions applied in the practice, due to the considerable resistance of the eggshell membrane against spray penetration.

The past three decades have seen a tremendous and world-wide increase in the production and application of chemicals, among them pesticides. Goulding (1975) estimated the new organic and inorganic compounds produced per year at 200,000. The increasing use of pesticides has resulted in a need for predicting hazards to wildlife, especially to useful birds living in ploughland.

The teratogenic effects of pesticides are of great importance (Hayes, 1975).

The teratogenicity of the organophosphate derivatives in chick and quail embryos has been investigated in detail (Clegg, 1964; Meiniel, 1977, 1978), but little information refers to the pheasant. As pheasant is a non-target, it is of importance to assess its susceptibility to teratogenic and/or fetotoxic effects of plant-protective substances.

This paper reports comparative teratological studies of Methylparathion 18 WP and Wofatox 50 EC in Japanese quail (*Coturnix coturnix japonica*) and pheasant (*Phasianus colchicus mongolicus et torquatus*) embryos.

Materials and methods

Treatment

Methylparathion 18 WP (M 18 WP) and Wofatox 50 EC (W 50 EC), containing 18 and 50% methylparathion as active ingredient, respectively, were the test pesticides used throughout the experiments.

A total of 469 and 252 fertile eggs from Japanese quails and pheasants, respectively, were assigned to one control and three treated groups per test material (see Table I) and incubated in a Ragus and a National type incubator under optimum environmental conditions (Kiss, 1973; Nagy, 1971).

The pesticides were suspended (M 18 WP) or emulsified (W 50 EC) in water and diluted to three various dose levels, of which the intermediate levels corresponded to the concentrations used in the field as spray (Registration document, 1976; Kónya, 1980). The applied concentrations were 0.05, 0.5, and 5.0% for M 18 WP and 0.02, 0.2, and 2.0% for W 50 EC.

Egg treatments were carried out around the middle of the normal incubation period, i.e., on the 9th day of the 16-day incubation period of the quail and on the 12th day of the 25-day incubation period of the pheasant. The pesticidal suspensions and emulsions were administered by a single injection into the air cell and in a volume of 0.05 ml/quail egg and 0.1 ml/pheasant egg. Control eggs received the same volume of distilled water. On the basis

Table I

Experimental data for the teratological study of Methylparathion 18 WP and Wofatox 50 EC

	Treatment on day of hatching		Evaluation on day of hatching		Dose mg/kg			No. of eggs evaluated	
	Quail	Pheasant	Quail	Pheasant	I	II	III	Quail	Pheasant
						Quail			
Methylparathion 18 WP	9	12	14	23	2.8	28	280	232	127
						Pheasant			
					1.7	17	170		
						Quail			
Wofatox 50 EC	9	12	14	23	2.25	22.5	225	237	125
						Pheasant			
					1.35	13.5	135		

of the average egg weight (9 g and 30 g, respectively), the doses of the organophosphates in mg/kg egg weight were similar at each corresponding dose level (see Table I).

The applied injection route was not hazardous for the developing embryo and provided a precise dosing.

Evaluations were conducted on days 14 and 23 of incubation, respectively.

Gross examination

At autopsy, all eggs were opened, and examined for gross lesions (teratogenicity), and for the ratio of viable to dead embryos, before and after treatment (embryo toxicity), and body weights were measured.

Skeletal deformities were identified in preparations stained by the method of Simons and van Horn (1971). As skeletal changes provide an important index in experimental teratology (Tompa et al., 1971), 3 embryos in each group (a total of 48 samples) were processed with this technique to evaluate skeletal defects and ossification.

Histological examination

Ossification was histochemically examined by measuring the alkaline phosphatase enzyme activity in the osteoblasts. For this purpose, the cervical vertebrae were sampled from 3 embryos per group, for the highest incidence of postural anomalies occur in the head and the neck. Spine samples, 2 to 5 vertebrae in one block were fixed in 80% alcohol at 4 °C, dehydrated in absolute acetone, and embedded in paraffin via benzene (Imre, 1981). Serial cross sections, 10 μm thick, were prepared by the MPSz 2 type microtome. The alkaline phosphatase enzyme activity was determined in identical segments of the vertebrae by using the method of Gömöri (1952) and Takamatsu (1939).

The ultrastructure of the embryonic cartilaginous tissue was examined in quail embryos that had received W 50 EC. Samples from the abnormal cervical vertebrae were fixed in Karnowsky's (1965) fixative mixture, post-fixed in 1% osmium tetroxide, dehydrated in a series of graded alcohol, and embedded in Durcupan-ACM resin (Fluka) (Palade, 1962). Sections were cut by the Reichert ultramicrotome. The electron micrographs were prepared in a Tesla BS 613 and a JEOL 100 S type electron microscope.

To determine cholinesterase enzyme activity of the apparently affected cervical muscles (m. longus colli, m. complexus major) at the motor end-plates, 3 quail and 3 pheasant embryos were sampled from each dose group and processed with the histochemical method developed by Koelle (1951) and Gerebtzoff (1953).

For light microscopic study of teratogenic and/or fetotoxic effects in the embryonic organs and tissues, additional 3 quail and 3 pheasant embryos were sampled in each dose group. Whole quail embryos stripped of feathers and the major organs from pheasants (heart, lungs, kidneys, liver, skeletal and cervical muscle) were fixed in 4% neutral formalin, embedded in paraffin, cut into sections 5 to 7 μm in thickness, and stained with haematoxylin–eosin and Azan (Kiszely and Barka, 1958).

Statistical analysis

Biometrical analysis included the chi-square test, Student's *t* test, and variance analysis (Elandt-Johnson, 1971; Finney, 1952, 1972; Fisher, 1926).

Results

Gross examination revealed that M 18 WP significantly increased the developmental abnormalities in the quail embryos that had received the highest dose. The other parameters were apparently not affected. At the dilution used in the field practice neither teratogenic nor fetotoxic effects (dwarfism, embryo mortality) were observed (Table II).

Table II

Review of the teratological study of Methylparathion 18 WP in Japanese quail embryos

Dose mg/kg	No. of fertile eggs	No. of viable embryos	Embryo weight (g)* $\bar{x}\pm$S.D.	Mortality before treatment	Mortality after treatment	No. of deformed embryos*
Control	60	52	3.70±0.49	4	4	2
2.8	61	52	3.69±0.47	1	8	7
28.0	57	49	3.97±0.29	5	3	4
280.0	54	43	3.58±0.38	3	8	19[d]

* referring to viable embryos; [d] $P < 0.01$, vs control

W 50 EC produced in quails similar incidences of developmental defects at each dose level and a significantly ($P < 0.01$) increased embryo mortality at the highest level. Viable embryos showed, therefore, decrease in number and the body weight of the survivors also tended to decrease ($P > 0.05$). At the practical dilution, the increase of embryo mortality was not significant, whereas the incidences of developmental abnormalities showed a significant ($P < 0.05$) increase, compared to the controls (Table III).

Table III

Review of the teratological study of Wofatox 50 EC in Japanese quail embryos

Dose mg/kg	No. of fertile eggs	No. of viable embryos	Embryo weight (g)* $\bar{x}\pm$S.D.	Mortality before treatment	Mortality after treatment	No. of deformed embryos*
Control	54	47	3.05±0.47	4	3	0
2.25	64	50	3.14±0.46	12	2	5[b]
22.5	64	57	3.04±0.52	3	4	5[b]
225.0	55	20	2.89±0.34	3	32[d]	5[d]

* referring to viable embryos; [b] $P < 0.05$; [d] $P < 0.01$, vs control

Pheasant embryos that had received M 18 WP elicited a dose-related significant ($P < 0.01$) depression in body weight at each dose level. Embryotoxic effects were not noticed at any dose, but the numbers of deformed embryos were significantly ($P < 0.02$; $P < 0.01$) higher than the control values, in each group treated, even at the level tenfold lower than the dilution used in the practice (Table IV).

Table IV

Review of the teratological study of Methylparathion 18 WP in pheasant embryos

Dose mg/kg	No. of fertile eggs	No. of viable embryos	Embryo weight (g)* $\bar{x}\pm$S.D.	Mortality before treatment	Mortality after treatment	No. of deformed embryos*
Control	31	31	18.12±0.72	0	1	0
1.7	32	32	17.42±0.48[d]	0	0	9[c]
17.0	31	29	17.56±0.32[d]	0	2	18[d]
170.0	33	28	16.30±0.12[d]	0	5	28[d]

* referring to viable embryos; [c] $P < 0.02$; [d] $P < 0.01$, vs control

W 50 EC caused a depression in the number of viable pheasant embryos at the highest dose. Embryo weight showed a significant ($P < 0.01$) increase at the intermediate dose and a significant ($P < 0.01$) reduction at the highest level. Following treatment, incidences of dead embryos and of developmental deformities were significantly ($P < 0.01$) over the control only at the highest dose, i.e. at the level tenfold greater than the dilution used in the practice. At this concentration, no evidence of teratogenic and lethal effects was observed in pheasant embryos (Table V).

Table V

Review of the teratological study of Wofatox 50 EC in pheasant embryos

Dose mg/kg	No. of fertile eggs	No. of viable embryos	Embryo weight (g)* $\bar{x} \pm$ S.D.	Mortality before treatment	Mortality after treatment	No. of deformed embryos*
Control	29	27	15.58 ± 0.90	0	2	3
1.35	32	31	15.45 ± 0.41	0	1	2
13.5	33	33	16.21 ± 0.32^{d}	0	0	3
135.0	31	19	14.41 ± 0.75^{d}	0	12^{b}	19^{d}

* referring to viable embryos; ^{b} $P < 0.05$; ^{d} $P < 0.01$, vs control

Among the anomalies produced by the two organophosphate derivatives (Table VI), cervical lordosis and scoliosis, and pes debilis were the most frequent in both avian species (Fig. 1). Anophthalmia developed only in quails and in only two cases for both pesticides. Rudimentum oris and microphthalmia were induced by W 50 EC, in identical numbers among quail and pheasant embryos. The incidence of thoraco-gastroschisis was twice as high in pheasants as in quails. Osteomalacia cranii developed only in quails, in two embryos altogether. Open umbilicus and hernia cerebri occurred only among quail embryos which had received M 18 WP.

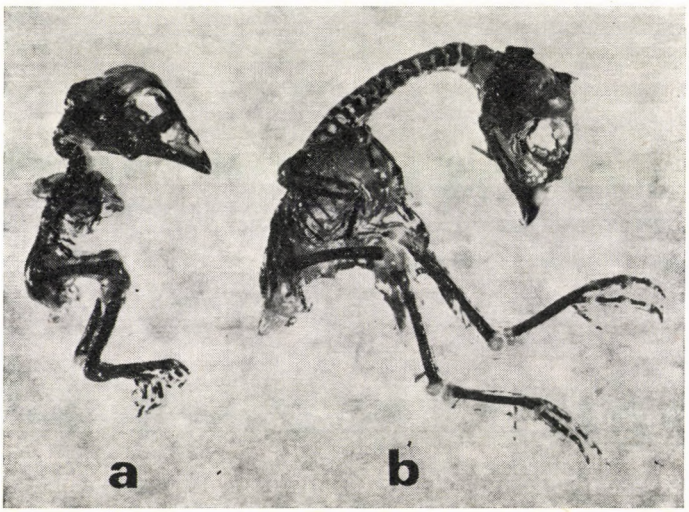

Fig. 1. Representation of 23-day pheasant embryos. *a)* retarded embryo after 135 mg/kg Wofatox 50 EC; *b)* control. Alizarin red and Alcian blue staining, ×1.5 (Photo: S. Kiss)

Under the experimental conditions, skeletal defects were not caused by either of the pesticides.

The alkaline phosphatase enzyme activity in the osteoblasts of the cervical vertebrae agreed with that of the controls.

The cholinesterase enzyme activity of the cervical muscles for all groups appeared intense, except for the pheasants that had received the highest dose of W 50 EC, in which case numerous motor end-plates were inhibited.

Table VI

Gross developmental abnormalities induced by Methylparathion 18 WP and Wofatox 50 EC in Japanese quail and pheasant embryos

Classification of abnormalities	Methylparathion 18 WP		Wofatox 50 EC		Control	
	Quail	Pheasant	Quail	Pheasant	Quail	Pheasant
Lordosis and scoliosis	8	55	5	17	0	0
Anophthalmia	2	0	2	0	0	0
Microphthalmia	0	0	1	1	0	3
Pes debilis	20	54	5	24	2	0
Thoraco-gastroschisis	1	0	2	4	0	0
Open umbilicus	9	0	0	0	0	0
Rudimentum oris	1	0	2	2	0	0
Osteomalacia cranii	0	0	2	0	0	0
Hernia cerebri	1	0	0	0	0	0
Exophthalmus	0	0	0	2	0	0

Staining of the end-plates showed also a considerable variation within the same cervical muscle.

The electron micrographs for quail embryos revealed no differences between controls and W 50 EC treatments in the ultrastructure of the cartilaginous tissue of the cervical vertebrae.

Light microscope findings exhibited degenerative changes to varying degree in the cervical muscle (m. longus colli) from quail and pheasant embryos given the higher pesticidal doses. Frequently, the lesion was unilateral. The affected muscles from pheasants showed numerous atrophied and hyperplasic muscle fibres of varying size (Fig. 2). The lesion in quails resembled that of pseudohypertrophia lipomatosa musculorum: the necrotic and atrophied muscle fibres were replaced by fat tissue (Fig. 3). The most striking degeneration was developed in the pheasants which had received the highest W 50 EC dose. Quail and pheasant embryos, considerably retarded in growth compared to the controls, exhibited hypoplasic changes of the cervical muscle.

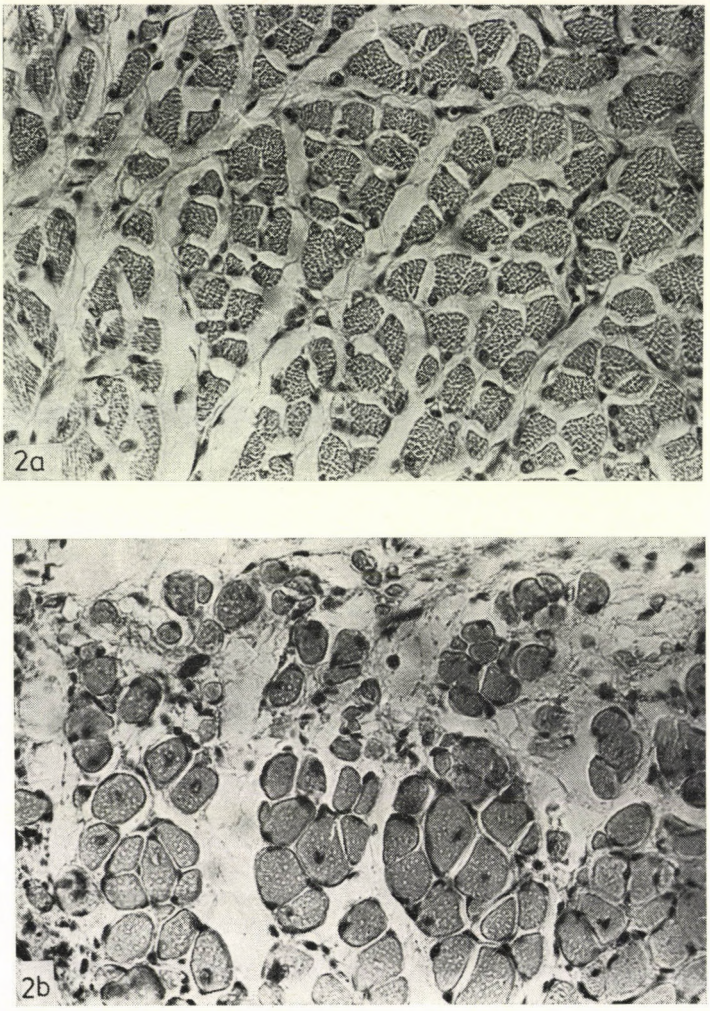

Fig. 2. Cervical muscle, transverse section, from 23-day pheasant embryos. *a)* control; *b)* treated with 135 mg/kg Wofatox 50 EC, showing severe atrophy and necrosis of the fibres. H. E. ×320

Discussion

One of the most critical phases in embryonal development is the period of tissue differentiation. Most of the teratogenical studies have, therefore, been conducted at this stage, i.e. in chick embryos on days 0–4 of incubation. The developmental abnormalities reported for parathion-exposed chick embryos of this stage included lordosis, scoliosis, reduction and adhesion of the vertebral bodies, pes debilis, thoraco-gastroschisis, atrophy or hyperplasia of the

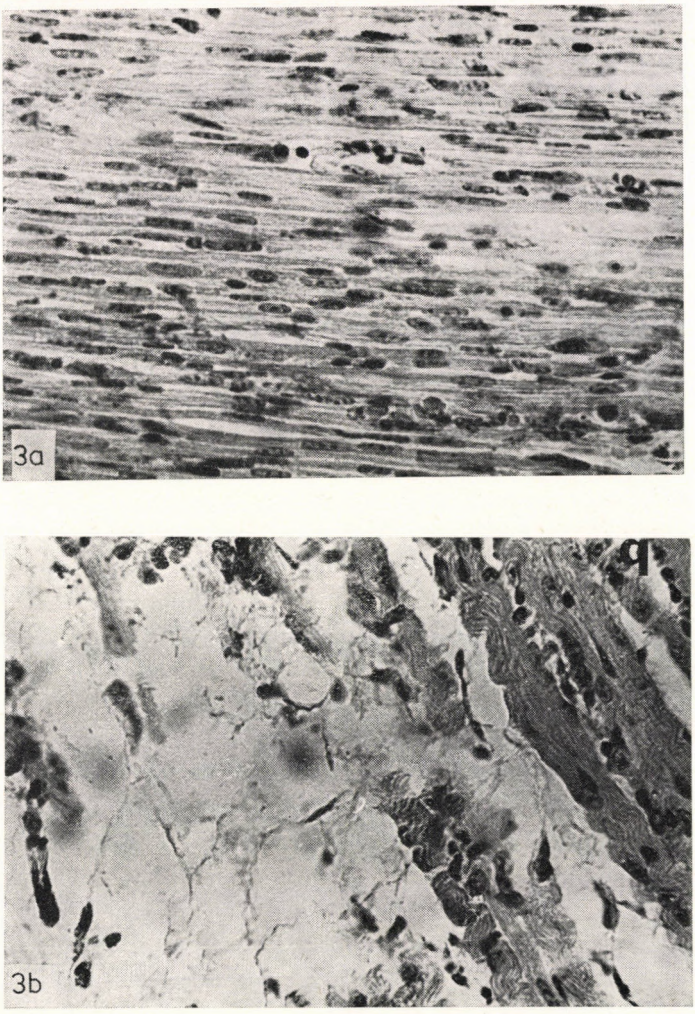

Fig. 3. Cervical muscle (m. longus colli) from 14-day quail embryos. *a)* control; *b)* treated with 225 mg/kg Wofatox 50 EC. The atrophied muscle fibres are replaced by fat tissue. H.E. ×320

cervical and tibial muscles, etc. (Iseki and Yamada, 1966; Meiniel, 1977; Roger, 1968).

Another critical phase in embryonal development is the onset of embryonic ossification and growth. Based on literary data (Terepka et al., 1971) and our experiences, this corresponds to days 9 and 10 of incubation in the quail and days 12 to 14 of incubation, i.e. the same time as for the chick embryo, for the pheasant (Fujioka, 1955; Murphy et al., 1957). Hereafter begins active calcification of the embryonic skeleton due to mobilization of eggshell calcium,

and this is followed by umbilical closing. The eyes, the beak, the feet, etc. acquire their final form at the same time (Kovács and Fehér, 1966).

The embryos in the present experiments were exposed to M 18 WP and W 50 EC at the intense period of embryonal ossification and growth.

Based on these data the following conclusion can be drawn:

— With identical active ingredient, the teratogenic and/or embryotoxic effects were concentration dependent. Methylparathion, of 18% active ingredient, proved to be teratogenic for both the quail and the pheasant embryos, whereas the Wofatox formulation containing 50% active ingredient showed embryotoxicity, besides teratogenicity (Tables II to V).

— The pheasant was considerably more susceptible than the quail to the teratogenic effects of M 18 WP. The developmental defects displayed by the viable embryos occurred around threefold as frequently (62%) in pheasants as in quails (21%). A single dose producing developmental abnormalities was 1.7 mg/kg for the pheasant vs. 280 mg/kg for the quail.

— Among the anomalies seen, lordoscoliosis, pes debilis and umbilical hernia were the most frequent, with an incidence ranging from 27 to 100%. The other developmental deformities (microphthalmia, rudimentum oris, etc.) occurred in 3 to 17% altogether. Embryonic weights were reduced by 5 to 10% at the high pesticidal levels, compared to the controls.

Contrary to the literary data (Iseki and Yamada, 1966; Meiniel, 1977; Roger, 1968), neither vertebral adhesion, nor extremital reduction alone, could be demonstrated in the skeletons of the treated avian embryos. This is in accordance with our previous biochemical findings. M 18 WP and W 50 EC failed to produce a definite and significant variation in serum alkaline phosphatase, calcium, and inorganic phosphor levels when blood samples from quail and pheasant embryos were used that had received similar treatments at appropriate age (Bartalits et al., 1981; Várnagy et al., 1981). These results might suggest that skeletal defects during intensive embryonal growth and ossification might be influenced by other biochemical factors not studied here (hormones, vitamins, enzymes, etc.).

In agreement with other reports (Meiniel, 1977, 1978; Upshal et al., 1968), the Wofatox formulation of 50% active ingredient produced in the cervical muscles partial inhibition of the cholinesterase activity at the motor end-plates of the pheasant and atrophic or hypoplasic changes in the cervical muscles of both avian species. As spinal vertebrae appeared normal, the potentiality of the organophosphates for inhibiting embryonic innervation and, as a consequence, muscular atrophy should also be considered among the factors leading to lordoscoliosis. It is likely that such a state may also induce abnormal distortion and curving of the neck.

In conclusion, our previous analytical (Várnagy and Füzesi, 1979) and teratological studies using the egg immersion route (Várnagy, 1980), as

well as the present results demonstrate that M 18 WP and W 50 EC are not expected to be teratogenic and/or embryotoxic pesticides under the usual practice of plant protection. The amount at which the organophosphate sprays may reach the developing embryo via the eggshell is around one eighthundredth of that injected into the air cell here.

Acknowledgement

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AN ANATOMICAL STUDY OF THE TENDON OF THE EQUINE BICEPS BRACHII MUSCLE

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The tendon running centrally through the belly of the m. biceps brachii and the lacertus fibrosus were studied post mortem for formation and function in 4 equine fetuses, 5 neonatal foals and 91 adult horses aged 1–23 years. Surgical transection of the m. biceps brachii's tendon and lacertus fibrosus was performed in four adult horses for in vivo study of disturbances in muscle function.

It has been shown that the tendon running through the belly of the m. biceps brachii represents neither a fascial thickening, nor a septum intramusculare, but a direct continuum of the muscle's tendon of origin. The tendon in question and, through it, the muscle itself, attaches on the tuberositas radii by a single tendon of insertion. A longitudinal torsion of the tendon in the muscle belly accounts for diminishing concussion and greatening tensing. We therefore propose that the tendon running through the m. biceps brachii, generally referred to as the tendinous intersection of that muscle, be named "tendo scapuloradialis", for precise reference to its formation, passage, and function.

The surgical modeling of the rupture of the above tendon showed that the fibres of the pennate muscle belly can partly overtake function during rest, but the coordination of the shoulder and elbow joints becomes less synchronous. Transection of the tendon was found to delimit forward progression in motion.

On transection of the m. biceps brachii, the extension of the elbow joint increased to such a degree that the hoof could be drawn up almost to knee level also without transection of the lacertus fibrosus. This observation is of diagnostic importance for surgical approach to the correction of ruptures of the above kind.

The lacertus fibrosus cannot be regarded as the exclusive continuum of the central tendon running through the m. biceps brachii, nor as the lateral tendinous portion of the muscle. It is in fact composed of one central lamina and two lateral laminae, of which only the central one is of tendinous origin, whereas the lateral laminae derive from a fascia and make up four fifths of the lacertus fibrosus's entire thickness in the adult horse. Transection of the lacertus fibrosus accounted for a light trouble in bearing weight.

The formation and function of the tendon running through the belly of the equine m. biceps brachii, and of the lacertus fibrosus converging from the latter muscle to the m. extensor carpi radialis have been differently interpreted by veterinary anatomists. The conflicting views have been responsible for occasional misdiagnosis and miscorrection of partial or complete ruptures of the m. biceps brachii.

Certain authors describe the tendon running through the muscle belly and the lacertus fibrosus as thickened fasciae (Krüger, 1929, 1931; Schauder, 1932; Zimmermann and Zimmermann, 1939) whereas others (Kovács, 1962; Fehér, 1980) have suggested that a septum intramusculare, converging to the belly of the m. biceps brachii from the brachial fascia, gives off the tendon-

like fascial bundle known as lacertus fibrosus, which then continues in the m. extensor carpi radialis, and attaches on the tuberositas ossis metacarpalis.

Again others (Kadletz, 1932; Ellenberger and Baum, 1974; Nickel et al., 1968; Sisson and Grossman, 1953; Taylor, 1959; Koch, 1960) have regarded the tendinous bundle as a continuum of the tendon of origin — Koch even refers to it as the central tendon of the m. biceps brachii — which divides shortly before the insertion of the muscle into a short medial and a longer lateral (lacertus fibrosus) portion, which furnish two insertions for the m. biceps brachii. Barone (1968) has suggested that a fibrous bundle (corde fibreuse) representing an elongation of the proximal tendon, runs through the m. biceps brachii, and detaches distally a short tendon of insertion and the lacertus fibrosus.

We investigated the morphological and functional aspects of the formation of the m. biceps brachii's tendon running through the belly and performed experimental studies on their function and dysfunction in the resting and moving horse. These studies have convinced us that the structure generally referred to as the tendon of the equine m. biceps brachii is anatomically and functionally a tendon, and should be re-named accordingly to prevent misinterpretations resulting from nomenclatural confusion.

Materials and methods

The structures known as tendon and lacertus fibrosus of the m. biceps brachii were studied post mortem in 4 equine fetuses, 5 newborn foals and 91 adult horses of different ages; of the latter 43 had been sports horses and 48 draught horses. All sports horses and two thirds of the work horses were of Eastern races, the remaining one third of the work horses descended half by half from Eastern and Western races.

The function and role of the tendon running through the muscle belly and lacertus fibrosus were studied at rest and in motion, by observing the dysfunction after transection of both structures in the right forelimb of four horses. The horses were 9–10 years old, as judged by their dentition, and weighed 400–700 kg. The surgical intervention was performed in intravenous chloralhydrate anaesthesia (10% solution, 8 g/100 kg) with the animal recumbent on its left side, according to the so-called Danish technique. In two horses the lacertus fibrosus was exposed and transected through a longitudinal cutaneous incision made in the elbow region. In the other two horses the tendon running through the belly was explored in the mid-region of the m. biceps brachii, through the m. brachiocephalicus, and was transected without injuring the belly of the muscle. Primary healing of the surgical wound followed in four cases.

Results

The m. biceps brachii has a tendinous origin and ends in a tendon of insertion (Fig. 1). The tendon of origin is two fingerbreadths thick in the adult horse. Aponeuroses converging from the posterior and lateral faces of the muscle to its belly furnish attachment for muscle fibres. After detaching the aponeuroses, the central part of the tendon of origin enters the muscle belly in the form of an about one fingerbreadth thick, approximately cylindrical bundle, runs through it, blends with the tendon of insertion and molds on the radius (Fig. 2). The tendon of insertion also detaches several aponeuroses, which impart a pennate appearance to the m. biceps brachii. The structure generally referred to as tendinous bundle develops in prenatal life (by 7 months), and can thus be identified full-length, with insertion on the radius in the newborn foal (Fig. 3). It flattens craniocaudally, and is surrounded altogether by fleshy muscle tissue in the proximal third of the m. biceps brachii (Fig. 4), while after the central third of the muscle it undergoes a lateral torsion, so that its medial margin assumes a cranial position. Distally, the tendon is compressed mediolaterally between two faces of muscle (Fig. 5).

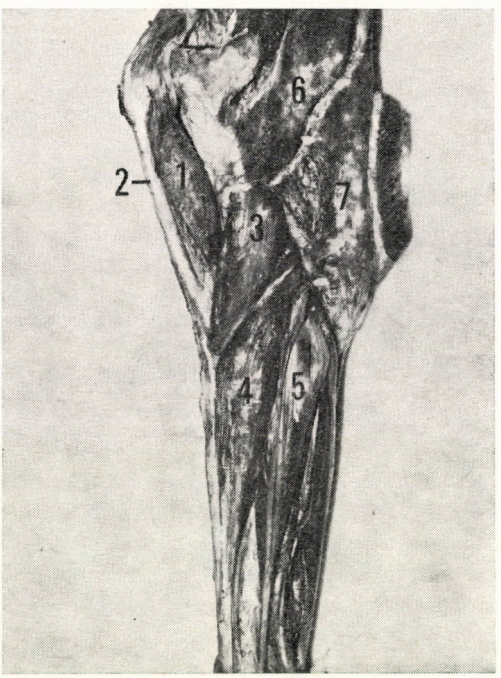

Fig. 1. M. biceps brachii of adult horse (lateral face; the lateral part of the belly is removed). 1, medial portion of the belly; 2, tendon running through the belly; 3, m. brachialis; 4, m. extensor carpi radialis; 5, m. extensor digitorum communis; 6, m. deltoideus; 7, m. triceps brachii

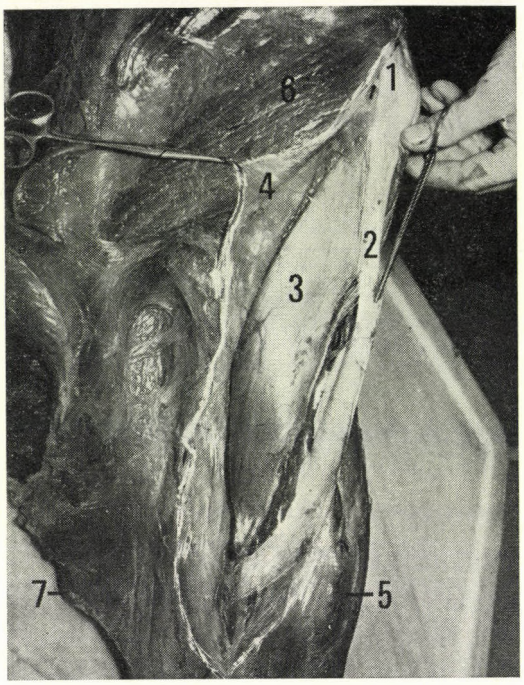

Fig. 2. Tendon running through the belly of the m. biceps brachii (craniomedial view; the medial portion of the muscle is partly removed). 1, tendon of origin; 2, tendon running through the belly; 3, medial portion of the belly; 4, outer fascial sheath (transected and inclined laterally); 5, m. extensor carpi radialis; 6, m. pectoralis profundus; 7, olecranon of the ulna

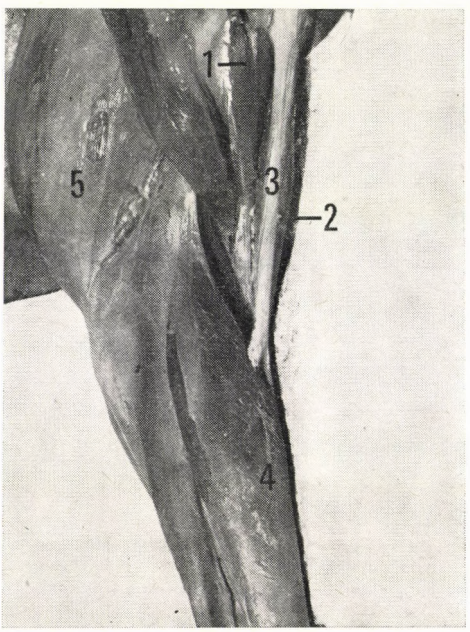

Fig. 3. M. biceps brachii of newborn foal (craniolateral view; the lateral portion of the belly is partly removed, partly inclined). 1, lateral portion of belly; 2, medial portion of belly; 3, tendon running through the belly; 4, m. extensor carpi radialis; 5, m. triceps brachii

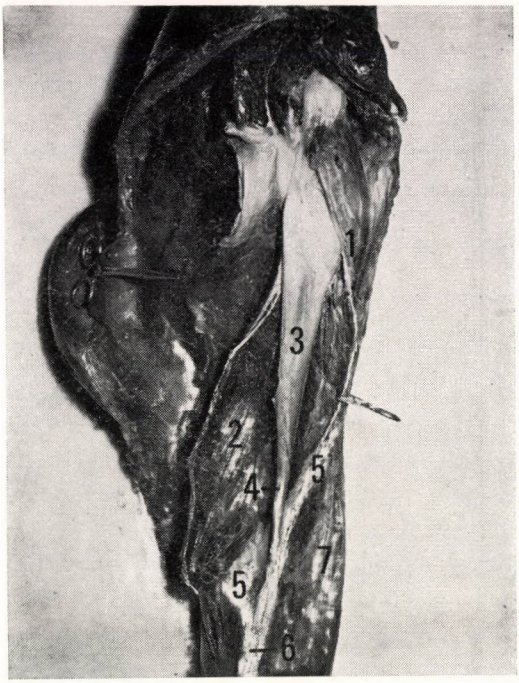

Fig. 4. The tendon running through the m. biceps brachii is surrounded by the belly of the muscle (cranial view; the belly is partly removed, partly inclined sidewards). 1, lateral portion of belly; 2, medial portion of belly; 3, tendon running through the belly; 4, cranially detached bundle of the tendon; 5,5, bilateral aponeuroses converging to the detached tendinous bundle; 6, lacertus fibrosus; 7, m. extensor carpi radialis

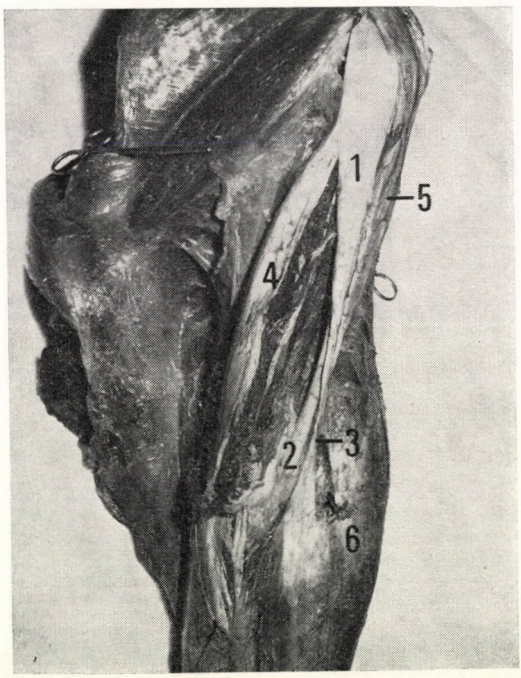

Fig. 5. Longitudinal torsion of the tendon running through the belly of the m. biceps brachii (craniomedial view; the medial portion of the belly is partly removed). 1, craniocaudal flattening of the tendon; 2, mediolateral flattening of the tendon; 3, cranially detached bundle of the tendon; 4, medial portion of belly; 5, lateral portion of belly; 6, m. extensor carpi radialis

Cranially, the tendon divides the m. biceps brachii into two unequal portions, a lateral and a medial one. The lateral portion covers one third, the medial portion two thirds, of the muscle's thickness. On the surface, the division of the muscle belly is indicated proximally only by a slight, craniomedially oriented oblique retraction, beneath which passes the tendon (Fig. 6).

The m. biceps brachii is invested by two fascial sheaths, an external and an internal one. The internal sheath wraps the muscle tightly to hold its divided parts in position. The fascial lamina of the internal sheath thickens to a strong aponeurosis, on the medial face proximally and distally, and on the lateral face only distally. The thickenings are already present in the equine fetus and newborn foal (Fig. 7). The tendon which turns into the muscle as a continuum of the tendon of origin blends distally with the aponeurosis running in the belly and on the face of the muscle, and forms with them a thick, strong tendon of insertion, which molds on the tuberositas radii (Fig. 8).

The tendon detaches at its anterior edge, from the centre of the muscle's middle third onwards, a triple, cranially narrowing tendinous bundle, which separates from it entirely at the distal end of the belly (Figs 5 and 9), and is joined bilaterally by the thick inner and outer aponeuroses of the muscle belly. The aponeurosis converging to the tendon from the medial portion is

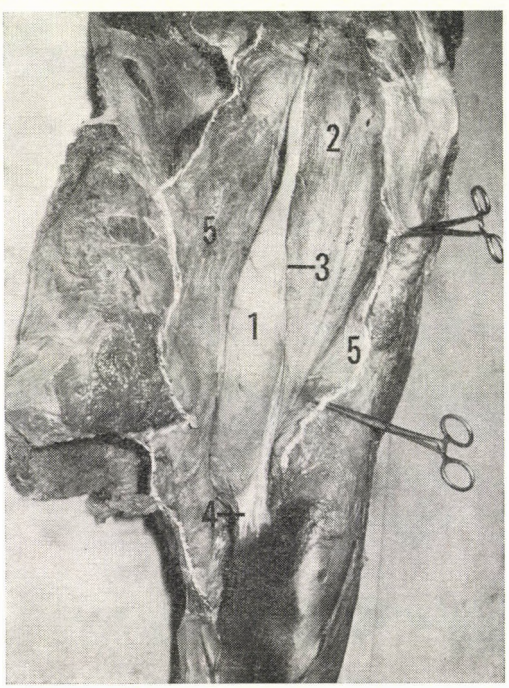

Fig. 6. Belly of the m. biceps brachii (craniomedial view; the outer fascial sheath of the muscle is transected and inclined to both sides). 1, medial portion of the belly; 2, lateral portion of the belly; 3, longitudinal fossa of the belly; 4, lacertus fibrosus; 5,5, outer fascial sheath

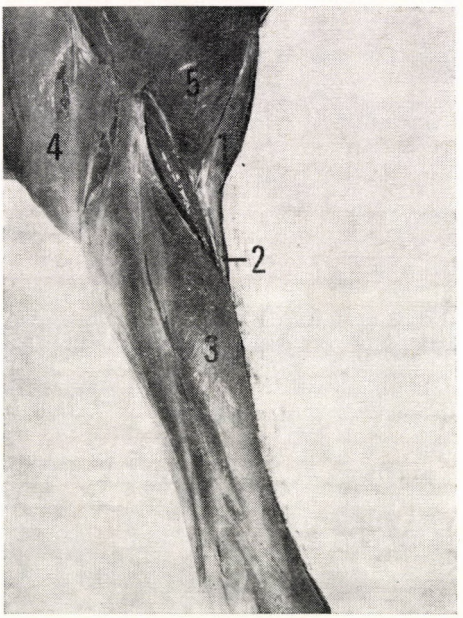

Fig. 7. Superficial aponeuroses of the m. biceps brachii in the newborn foal (craniolateral view; the outer fascial sheath is transected). 1, m. biceps brachii; 2, lacertus fibrosus; 3, m. extensor carpi radialis; 4, m. triceps brachii; 5, m. brachiocephalicus

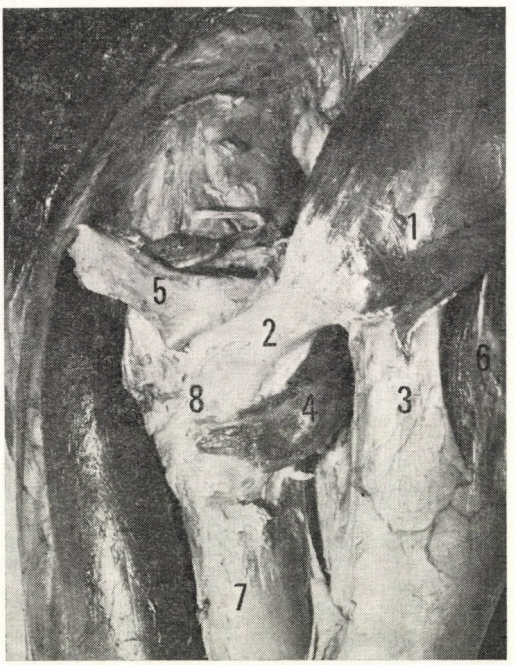

Fig. 8. Insertion of the m. biceps brachii on the tuberositas radii (medial face). 1, belly of the muscle; 2, tendon of insertion; 3, lacertus fibrosus; 4, m. brachialis; 5, ligamentum collaterale mediale (transected at attachment of insertion, and inclined); 6, m. extensor carpi radialis; 7, radius; 8, tuberositas radii

thinner than the one converging from the lateral portion. Thus the lacertus fibrosus arises from three laminae, a central one and two lateral ones; its margin is cranially sharp, and it converges distally before the elbow joint, on the medial surface of the m. brachialis, to the m. extensor carpi radialis (Fig. 10). The lacertus fibrosus is loosely inclosed by the fascial sheath of the m. biceps brachii; its terminal portion blends with the fascia of the forearm, and joins along with the latter the tendon of the m. extensor carpi radialis (Fig. 11). The three laminae forming the lacertus fibrosus can be distinguished already at 7 months of prenatal life. Thickening of the lacertus fibrosus with progressing age is due mainly to that of the lateral laminae, which make up four fifths of the lacertus fibrosus's entire thickness in the adult horse. The size of the lacertus fibrosus varies with race, body dimensions, age and use of the horse. The lacertus fibrosus of the adult horse is 35–55 mm wide and 3–8 mm thick.

On transection of the m. biceps brachii's tendon, no trouble in bearing weight was observed during rest, but the ipsilateral shoulder joint was by about 20° more open than the contralateral one (Fig. 12). With the forearm drawn backward, the extension of the elbow joint was by about 30° greater

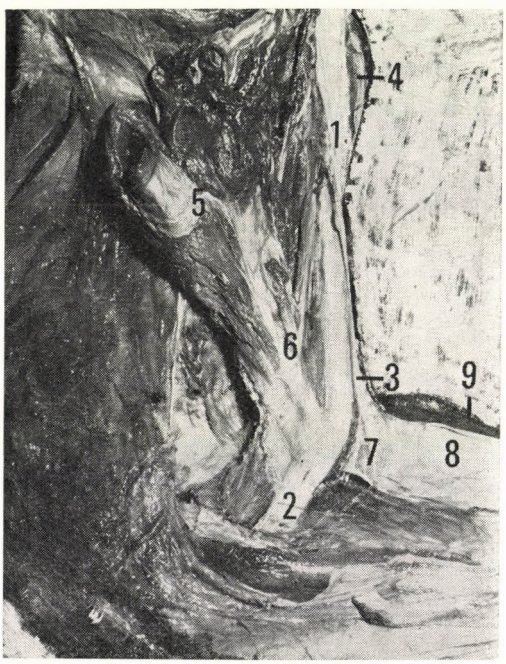

Fig. 9. Laminae joining in formation of the lacertus fibrosus (medial view; the medial portion of the belly is partly removed and inclined). 1, tendon running through the belly of the m. biceps brachii; 2, tendon of insertion of m. biceps brachii, converging to the radius; 3, bundle detached from the cranial portion of the tendon (central lamina of lacertus fibrosus); 4, lateral portion of belly; 5, medial portion of belly; 6, aponeurosis of the muscle belly converging to the tendon of insertion; 7, aponeurosis of the muscle belly converging to the detached tendinous bundle; 8, lacertus fibrosus; 9, m. extensor carpi radialis

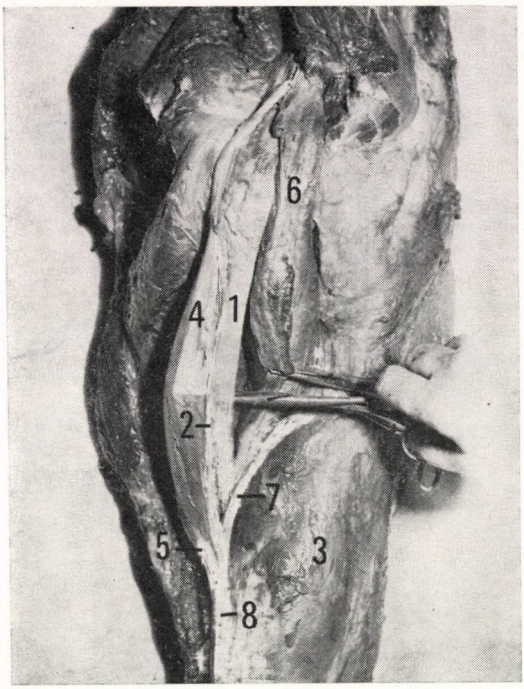

Fig. 10. Bilateral convergence of the aponeuroses of the muscle belly to the bundle detached from the tendon of the m. biceps brachii (craniolateral view). 1, tendon running through the belly; 2, detached bundle of tendon; 3, m. extensor carpi radialis; 4, medial portion of the belly and 5, its aponeurosis; 6, lateral portion of the belly (drawn sideward) and 7, its aponeurosis; 8, lacertus fibrosus

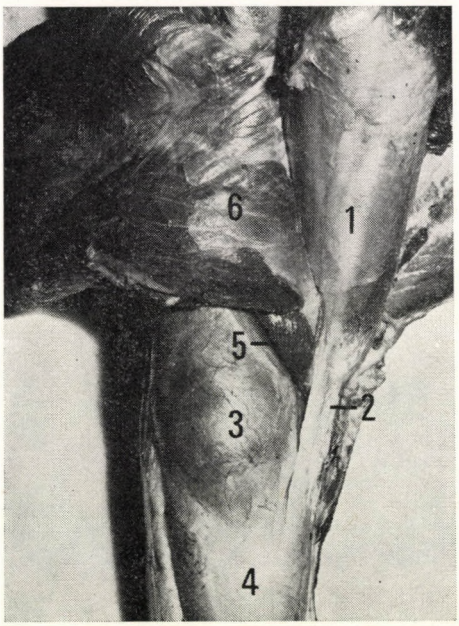

Fig. 11. Lacertus fibrosus (cranial view). 1, surrounded by the fascial sheath of the m. biceps brachii; 2, lacertus fibrosus; 3, m. extensor carpi radialis; 4, fascia antebrachii; 5, m. brachialis; 6, m. brachiocephalicus (drawn sideward)

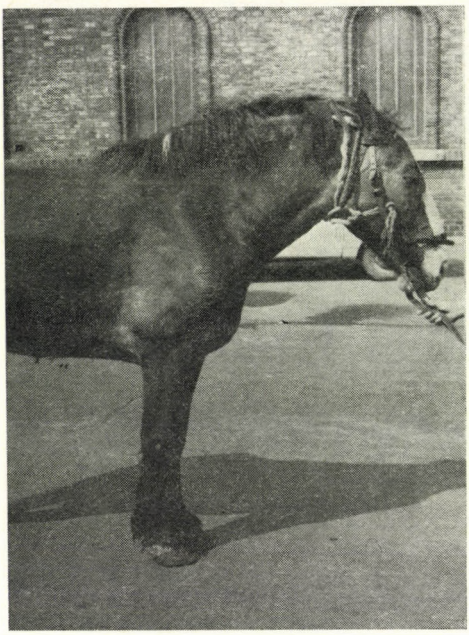

Fig. 12. Ten-year-old castrated male draught horse after transection of the tendon running through the belly of the m. biceps brachii on the right forelimb

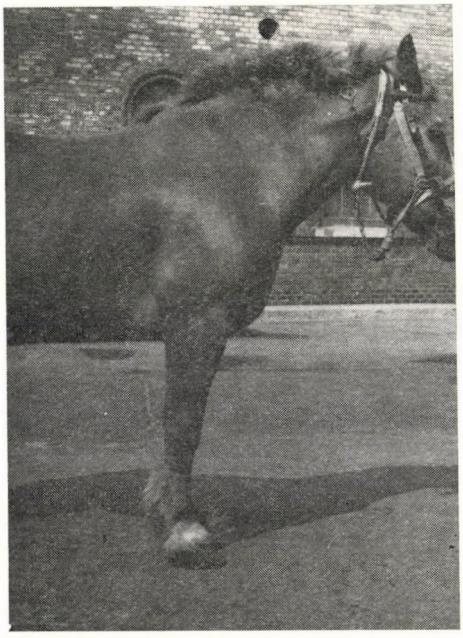

Fig. 13. Nine-year-old castrated male draught horse after transection of the lacertus fibrosus on the right forelimb

than in normal conditions; with the carpal and digital joints extended, the hoof could be lifted almost to the level of the tarsal joint. Extension of the elbow joint was followed by a lesser extension of the shoulder joint compared to the sound limb. During walk a slight lameness, marked by a light difficulty in extending, was observed in the operated anterior extremity. The operated thoracic limb was occasionally dragged along the ground, accounting for paring of the horn in the area of contact after 1–2 weeks. A slight aggravation of the lameness was observed during trot.

On transection of the lacertus fibrosus, there was no difference during rest between bearing weights of the operated and sound thoracic limb (Fig. 13). No change was observed either in the extension of the elbow and shoulder joints. During progression, a slight lameness was noticed, but the forward movement of the leg was hardly inhibited. Lameness was aggravated during trot.

Total transection of the m. biceps brachii and its tendon in the central region made possible an extreme extension so that, with the elbow joint extended, the limb could be drawn near to the knee joint. Subsequent transection of the lacertus fibrosus did not further increase the extension of the elbow joint.

Discussion

The present findings support the conception that the tendon running through the belly of the m. biceps brachii represents a direct continuum of the tendon of origin of that muscle, as suggested earlier by Nickel et al. (1968), Taylor (1959), Sisson and Grossman (1953), and Barone (1968). We have found that the tendon generally referred to as tendon is formed by part of the collagen fibre bundles of the broad tendon of origin, and the passage of these fibres can be followed up from the tuber scapulae to the tuberositas radii, on which they mold for insertion. We therefore disagree with the above-cited authors on the point that the tendon running through the belly would continue in a single tendon of insertion, which attaches the muscle on the radius. The tendon does not, in fact, divide into medial and lateral branches, whence the lacertus fibrosus cannot be regarded as the longer lateral tendinous portion of the m. biceps brachii.

We also disagree with Krüger (1929, 1931), Schauder (1932) and Zimmermann (1939), who described the tendon running through the muscle belly of the m. biceps brachii as a detached portion of the fascia brachialis. We have observed that, although the thickened deep portion of the muscle's fascial sheath does invest the tendon cranially, and the thick aponeurosis of the sheath does account for thickening of the latter at the medial edge, the aponeurosis, which blends with the tendon. does not join in the muscle's tendinous

insertion on the tuberositas radii, for it forms ultimately the two lateral laminae of the lacertus fibrosus.

A further reason why the tendon of the m. biceps brachii cannot be regarded as a fascial thickening is its well-developed condition in the fetal and neonatal horse. As a matter of fact, the fascial sheath portions converging to the tendon will thicken with progressing age and special use.

The tendon of the m. biceps brachii cannot be regarded as an intramuscular septum either, although it divides, incompletely, the muscle into two portions. One argument against its designation as a septum is its formation from the muscle's tendon of origin, and another argument is that it ends distally, without transition, as a tendon of insertion. With these facts in mind, the structure in question is anatomically and functionally a tendon, and we propose for it the name "tendo scapuloradialis", which refers not only to its nature, but also to its passage through the muscle and its function.

The m. biceps brachii fixes the shoulder joint and elbow joint of the horse. According to Zimmermann (1915, 1936, 1938, 1939), the tendon, which runs through the belly of the muscle, ensures the simultaneity of shoulder and elbow joints flexion. This function of the muscle presupposes the assistance of the tendon. The horse being a characteristically fast-moving animal, its m. biceps brachii necessarily possesses a central tendinous formation which furnishes fixing of the shoulder joint, coordinates the movement of the latter with the elbow joint, and facilitates standing. Our studies on the effect of transection of the tendon running through the m. biceps brachii have substantiated the above conclusion.

Postoperatively, there was no disturbance in the weight-bearing of the thoracic limb, because, although the tendon was transected, the fibres of the pennate belly remained intact and furnished the fixing of the shoulder joint with hardly more muscle work than normally. Function of the intact muscle belly can explain that no extreme flexion of the shoulder joint occurred during bearing weight.

The extension of the elbow joint was, however, increased to a certain degree during simultaneous extension of shoulder and elbow joint. Increased extension of the elbow joint, produced by drawing the forearm backward, was followed by a lesser extension of the shoulder joint than expected. This can be explained by absence of the action of the transected tendon, which, as said before, coordinates the movements of the two joints. The coordination of these joints was less complete after transection of the tendon. So it was possible that the extension of the shoulder joint was occasionally of a minor degree than before the operation.

Transection of the tendon accounted for progression trouble during walk. According to Zobundžija et al. (1977, 1978), the progression trouble gives rise to acceleration of movement.

In the living horse, complete rupture or transection of the m. biceps brachii accounts for a nearly horizontal position of the scapula and an almost complete openness of the elbow joint during rest. The elbow joint can be extended even further if the affected forelimb is drawn backward. According to Kómár (1960), B. Kovács and Tamás (1977), a rupture of the m. biceps brachii's lacertus fibrosus can additionally occur; in such cases the extension of the elbow joint is of such a degree that the extremity can be brought in an almost parallel position to the body, and the limb can be drawn up near to the level of the knee.

In the present study we were able to produce such an extreme extension of the elbow joint by total transection of the m. biceps brachii, with the lacertus fibrosus left intact. This circumstance should be taken into consideration for the diagnostic judgement of ruptures of the m. biceps brachii of the horse.

We have found that, as to its formation, the lacertus fibrosus is composed of one central lamina and two lateral laminae. The central lamina is of tendinous origin, being detached from the middle third of the tendon that runs through the m. biceps brachii, by separation of the tendon's mediolaterally flattened cranial part. The two lateral laminae are, however, of fascial origin, since they are formed by the muscle belly's external aponeuroses of attachment. Thus, the lacertus fibrosus is not just a continuum of the tendon running through the muscle belly or one of the muscle's two tendinous insertions, as has been erroneously proposed by several authors (Nickel et al., 1968; Koch, 1960; Sisson and Grossman, 1953; Taylor, 1959).

In the young, 1.5–2 years old horse the tendinous central portion makes up one third of the total thickness of the lacertus fibrosus. In the adult horse, however, functional thickening of the fascial sheaths, which takes place later in life (Gyűrű, 1981), accounts for more than four fifths of the total thickness of the lacertus fibrosus. The lacertus fibrosus is intimately associated with the m. biceps brachii, partly through the latter's central tendon, partly through the aponeuroses of the muscle belly, not only in adulthood, but already in fetal life. Indications of that association were observed earlier by Strubelt (1931), Zietzschmann (1925), Martin (1923) and Schmaltz (1928).

Unlike other authors, we observed a considerable alteration of stride during motion on transection of the lacertus fibrosus. A slight trouble in bearing weight of the operated limb accounted for a slight increase in extension and flexion compared to the sound limb. The lameness was more pronounced during trot than at rest. We attribute these changes to interruption of association between m. biceps brachii and m. extensor carpi radialis through transection of the lacertus fibrosus, and consequent failure of the tendon running through the m. biceps brachii to assist extension of the carpal joint by tensing.

Obviously, transection or rupture of the lacertus fibrosus also prevents the latter's cooperation in fixing the carpal joint, and affects adversely the coordination of the movements of shoulder, elbow and carpal joints.

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GROWTH PATTERNS OF LIMB BONES IN SWINE

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The authors determined and compared the length and ash content of limb bones (humerus, radius, ulna, metacarpus IV, femur, tibia, metatarsus IV) in Cornwall and Landrace pigs at different ages. The limb bones grew more rapidly in Cornwall pigs than in the Landraces till the age of 80–100 days. Later the limb bones in Landraces became considerably longer than those of the Cornwalls.

The relative bone ash was almost the same in both breeds. During the period around weaning, the bone ash values tended downwards in almost all the limb bones of both breeds.

Thirty per cent of the Landraces of 160-day-old and 100 kg weight were suffering lameness which can be considered a syndrome of bone weakness. The limb bones of these pigs both in size and relative ash content were nearly the same as those of the one-year-old, healthy Cornwalls which were not liable to lameness. In the authors' opinion, the development of lameness cannot be purely explained either by an inadequate growth of bones or the insufficiency of minerals.

Recently, besides the well-known limb end diseases in swine (panaritium, ragged pad, horn infraction), the bone and joint diseases have attracted an utmost attention. In spite of the investigations into the aetiology and the pathogenesis of the above disorders by a number of authors, the related questions have remained unanswered so far. The comprehensive name of the lameness which appears as a typical bound moving is "syndrome of limb or bone weakness" when the reason cannot be determined through the usual clinical examinations. The disease is accompanied by pain which suppresses appetite, lessens gain in weight and modifies the neurohormone balance. A neurohormone imbalance, naturally, may cause losses in breeding stocks. On the basis of observations, it can be stated that disorders in locomotion occur firstly in overbred swine of the meat type. Many authors (Behrens, 1961; Claus, 1962; Dämmrich and Unshelm, 1972; Discher, 1972; Kerk, 1974; Nielsen, 1973; Richter, 1958; Vaughan, 1971), attribute these changes, besides an inadequate constitution and the very quick muscular growth, to weakness of the skeleton system and to a slow development of cartilages and bones.

The purpose of the experiment described here was to furnish data both on the development of skeletal diseases and of disorders in locomotion. In order to answer the questions arising, we have made researches. Part of the results of these have already been published (B. Kovács and Suba, 1979; Szilágyi et al., 1978, 1979, 1980). In this study, we wish to point out whether the development of limb bones — the longitudinal growth and the quantity

of minerals in them — has a share in the development of disorders in locomotion or not. Up to now we have had no data on the rate of development of skeletal system in swine bred in Hungary.

We have chosen consciously (*i*) the Cornwall swine, which grows and develops slowly, has a good skeletal system and constitutional rigidity and does not give meat intensively, and (*ii*) the Landrace hybrid, which grows quickly, gives long chop and ham and has a good meat-producing ability.

Materials and methods

We examined 60 Cornwall pigs (aged 1, 5, 10, 20, 30, 40, 80, 160, 360 and 720 days) and 86 Landrace hybrids (aged 1, 5, 30, 40, 50, 70 and 160 days). In each age group, the sex ratio was 1:1. The following bones were studied: humerus, radius, ulna, metacarpus IV, femur, tibia and metatarsus IV. After being freed of muscle, the lengths of the bones were measured. Samples for chemical tests were cut from the central area of the bones (corpus). After fat extraction the samples were dried and burned to ash. The average length of bones is given in mm and the average ash content is expressed in relation to the fat-free weight (mg/g).

Results

The length and ash content of limb bones at different ages in Cornwall and Landrace pigs are given in Tables I and II, and in Figs 1–4.

Table I

Length (mm) of the examined bones in Cornwall

Age (days)	n		Humerus		Radius		Ulna	
	C	L	C	L	C	L	C	L
1	6	6	49.3	48.1	35.0	33.5	49.0	47.2
5	6	6	56.7	52.3	38.6	35.2	54.6	49.6
10	6	—	65.1	—	45.7	—	64.7	—
20	6	—	71.6	—	51.0	—	76.0	—
30	6	6	83.3	81.7	60.5	57.3	87.5	82.5
40	6	4	91.5	85.0	66.5	60.0	97.5	87.0
50	—	12	—	89.3	—	63.4	—	90.8
70	—	4	—	96.0	—	69.5	—	102.0
80	6	—	113.5	—	81.2	—	122.5	—
160	6	48	146.3	174.0	110.3	126.2	157.8	175.9
360	6	—	181.8	—	133.1	—	188.6	—
720	6	—	233.2	—	173.2	—	237.2	—

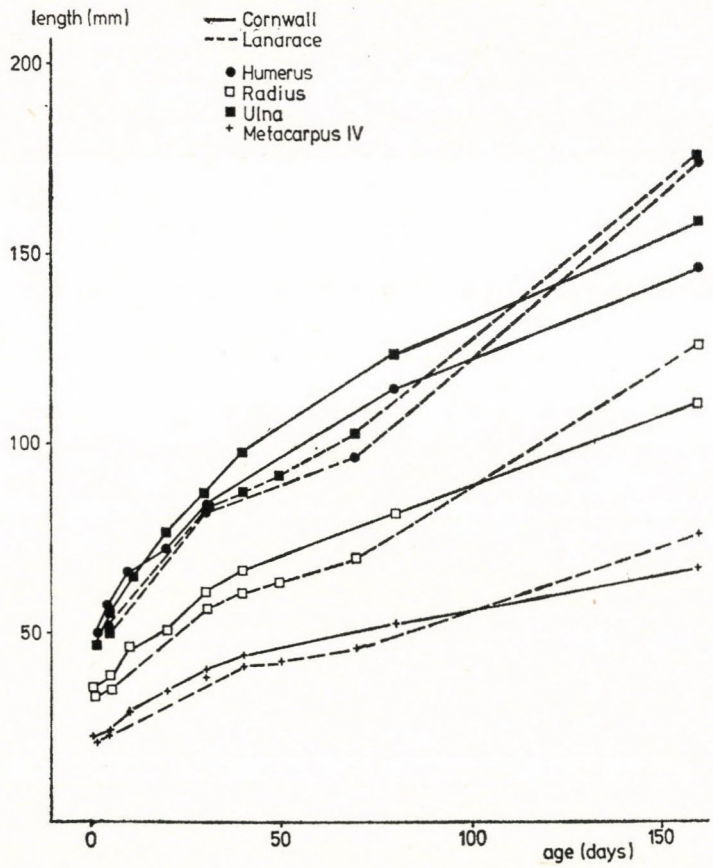

Fig. 1. The length of fore limb bones in Cornwall and Landrace pigs

(C) and Landrace (L) pigs of different age

Metacarpus IV		Femur		Tibia		Metatarsus IV	
C	L	C	L	C	L	C	L
22.2	21.5	48.5	48.0	44.6	44.5	24.2	23.4
23.8	23.2	56.6	52.3	50.6	47.7	27.0	24.9
28.8	—	66.0	—	60.6	—	32.7	—
33.9	—	75.3	—	68.5	—	37.0	—
39.8	38.5	91.0	85.3	82.3	77.7	45.0	42.9
43.6	41.0	99.0	92.0	88.2	82.3	49.7	44.3
—	42.1	—	96.4	—	87.5	—	47.7
—	45.6	—	110.0	—	99.0	—	54.0
51.8	—	127.6	—	114.2	—	59.6	—
66.6	75.5	169.8	192.3	156.7	177.6	77.6	85.5
80.0	—	209.6	—	191.1	—	85.9	—
86.0	—	252.7	—	240.2	—	96.3	—

Table II

Ash content (mg/g) of limb bones in Cornwall

Age (days)	n		Humerus		Radius		Ulna	
	C	L	C	L	C	L	C	L
1	6	6	545	553	509	545	499	512
5	6	6	552	556	538	537	494	516
10	6	—	551	—	503	—	501	—
20	6	—	560	—	502	—	494	—
30	6	6	565	556	529	521	479	481
40	6	6	564	563	496	501	413	481
50	—	12	—	573	—	508	—	487
70	—	4	—	575	—	523	—	498
80	6	—	591	—	525	—	502	—
160	6	48	617	618	544	595	534	559
360	6	—	637	—	566	—	560	—
720	6	—	647	—	592	—	584	—

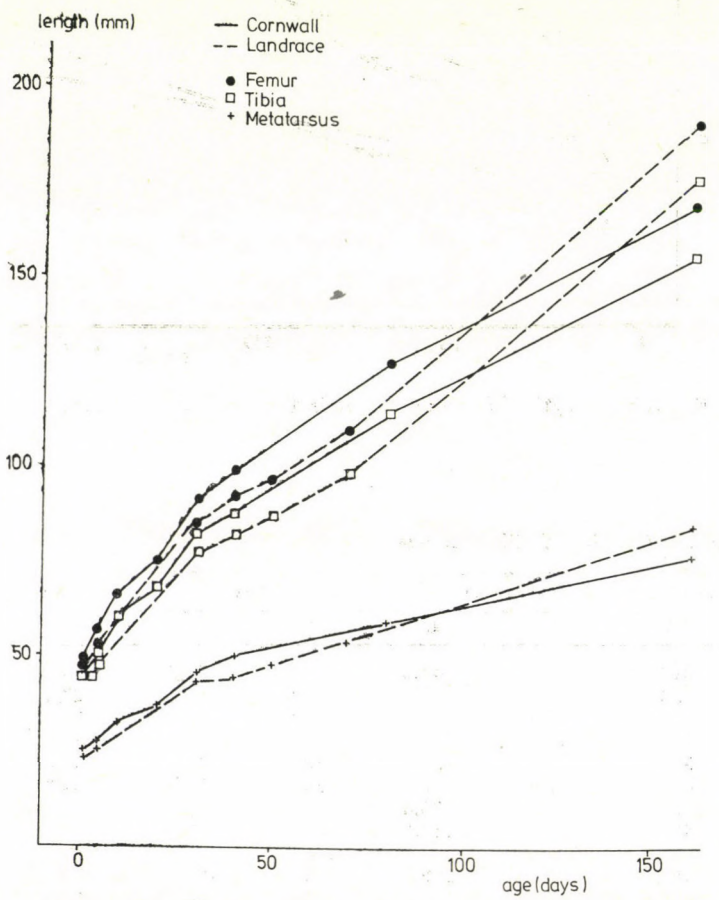

Fig. 2. The length of rear limb bones in Cornwall and Landrace pigs

(C) and Landrace (L) pigs of different age

Metacarpus		Femur		Tibia		Metatarsus	
C	L	C	L	C	L	C	L
431	501	541	573	523	549	417	508
400	479	548	574	535	552	397	481
472	—	547	—	520	—	463	—
459	—	548	—	531	—	459	—
497	443	559	578	542	534	502	460
528	476	547	572	534	535	528	471
—	475	—	561	—	546	—	483
—	488	—	570	—	551	—	512
514	—	560	—	553	—	515	—
558	559	586	638	583	593	572	572
575	—	613	—	614	—	583	—
583	—	628	—	623	—	599	—

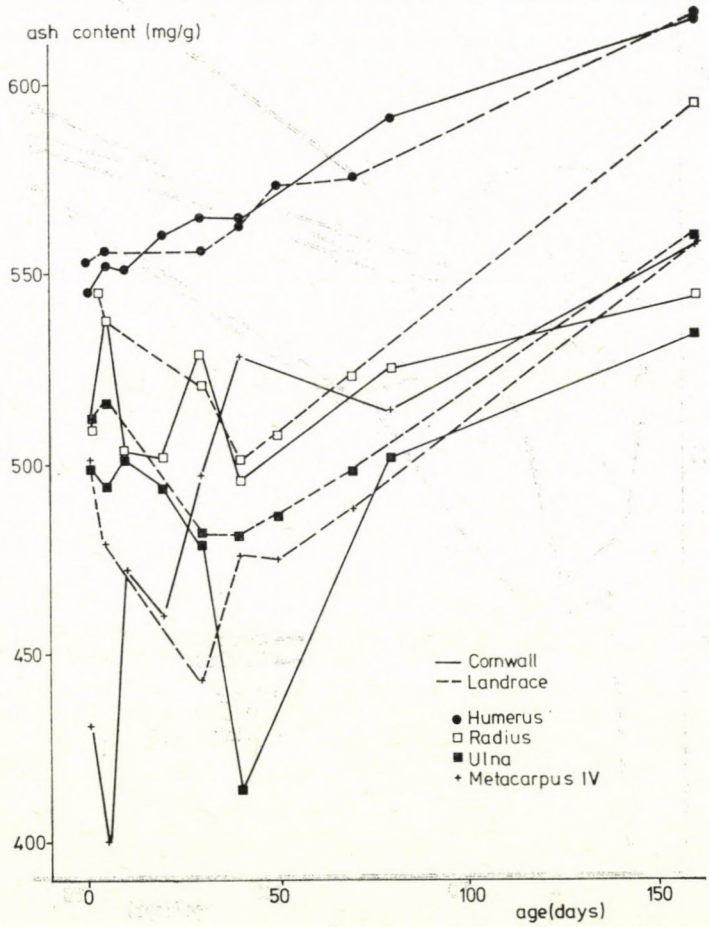

Fig. 3. Bone ash in fore limb bones of Cornwall and Landrace pigs (mg/g)

Humerus. In Cornwall pigs the humerus grew relatively quickly till days 30–40 but from this time on its growth tended to be less intensive. The growth of the Landrace pigs' humerus was relatively quick till the 30th day, slightly slower between days 30 and 70 and quicker again from day 70 on. The Landrace pigs' humerus was shorter than the Cornwalls' till the age of 70 days, however, at 160 days of age the formers' humerus was considerably longer than that of the Cornwall (Table I, Figs 1–4).

The bone ash of the humerus (Table II, Figs 3 and 4) remained practically unchanged in Landraces till the age of 30 days, then it increased consider-

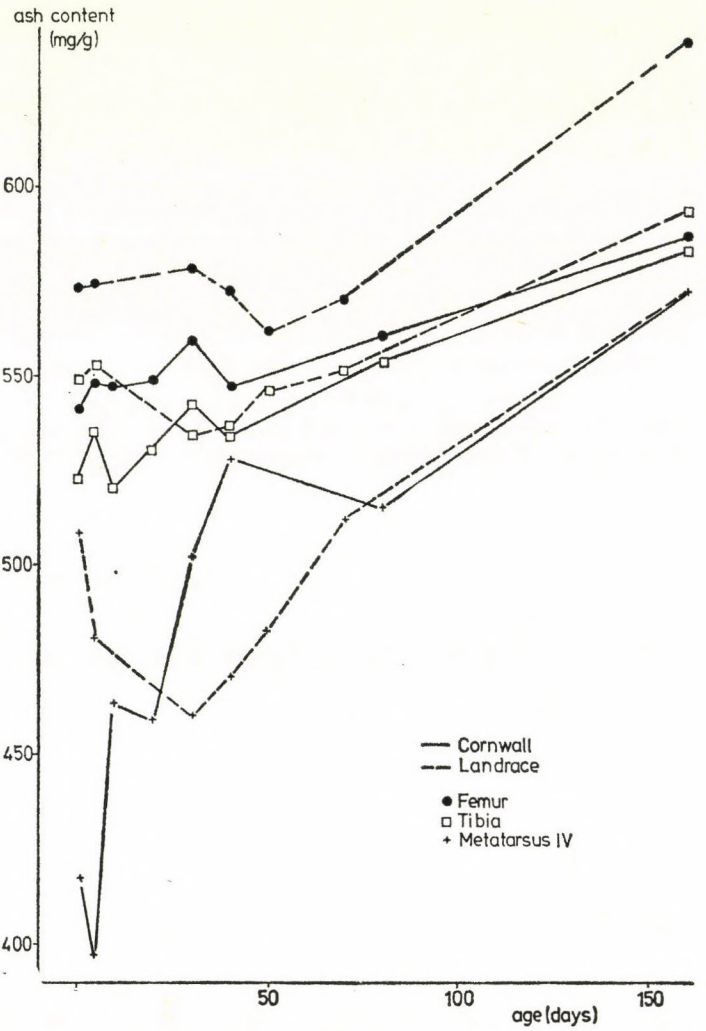

Fig. 4. Bone ash in the rear limb bones of Cornwall and Landrace pigs (mg/g)

ably. The bone ash in humerus of Cornwalls increased in proportion with the age, except for those of 40 days old, in which a slight decrease was observed.

Radius. The radius in both breeds grew intensely till day 30, then it stopped growing. This stoppage was less pronounced in the Cornwalls than in the Landraces and it was followed by an extremely quick growth only in the Landraces. The radius of the Landrace pigs was shorter than that of the Cornwalls till day 70, but at the age of 160 days it reached the size of the radius of 260-day-old Cornwalls. The growth rate can be well demonstrated by this example.

The relative ash content of the radius in 10, 20 and 40 days old Cornwall pigs was just the same or rather less than it was at the age of 1 day, consequently it was increasing with the age. The ash content of the radius in Landrace pigs decreased strikingly till day 40, then it began to increase, at a higher rate than in the Cornwalls.

Ulna. The growth of the ulna was rather quick till days 30–40 in the Cornwalls but later its rate slowered. In the Landrace pigs, the ulna was shorter only till the age of 70 days. At 160 days, it was already considerably longer.

The relative ash content of the ulna in Cornwall pigs hardly changed till days 10–20, but at this age it began to show a strong decrease and it had reached its lowest level by day 40. At the age of 80 days it was approximately the same as the relative ash content of the 1-day-old pig. After this, it increased with the age. The ash content of the ulna in Landrace pigs was usually higher than in the Cornwalls, however, at the age of 30–40–50 days a considerable decrease was observed in Landrace pigs too.

Metacarpus IV. The length of this bone was about the same for the pigs in both breeds till the age of 30 days. Later, in the Cornwalls it was growing steadily, while in the Landrace pigs first (at the age of 40, 50 and 70 days) a low growth rate, then an extremely intense lengthening was characteristic.

The bone ash level at the age of one day was considerably lower in the Cornwall pigs than in the Landraces and it was even lower at the age of 5 days, then it increased strikingly. After birth, the bone ash in this bone of Landrace pigs tended to decrease, too, and it reached its lowest level at the age of 30 days.

Femur. This bone grew steeply in both breeds till day 30. In Landrace pigs it grew at a slower rate at the age of 30–70 days; subsequently the growth was extremely quick. In Cornwalls the growth rate of the femur slowed down steadily.

The mineral content of the femur was higher for each age group in Landraces than in the Cornwalls. The bone ash displayed hardly any change till days 40–50, or it rather lessened at the age of 40–50–70 days in the Landraces. However, later it increased strikingly.

Tibia. The growth of this bone was very intensive for both breeds only till day 30. Till the age of 70–80 days, the tibia in Landraces was shorter than in the Cornwalls in each age group, while at the age of 160 days it was already longer by about 20 mm.

The bone ash showed hardly any change till days 30–40, in Landrace pigs it was even smaller, but in the following period the growth was intense and steady.

Metatarsus IV. This bone was shorter in each age group up to the age of 70–80 days in the Landrace pigs than in the Cornwalls, but then it lengthened quickly in the former, and became considerably longer than the same bone of the Cornwalls.

There was a great difference in bone ash between the two breeds in the first 5 days of life; this value was extremely high for the Landraces but then it showed a slight decreasing tendency till day 30. In the Cornwalls the bone ash level was rather low but continuously increasing.

Discussion

The longitudinal growth of fore and hind limb bones in Cornwall pigs was not linear. Their growth was rapid till days 30–40, moderate till day 160, then even slower, but it had not stopped growing even by the age of 720 days. Related to the length at birth, the hind limb bones grew more quickly than the corresponding bones of the fore limbs.

In spite of the fact that the Landrace hybrids grow quickly and intensely, their limb bones were shorter up to the age of 70 days than those of the Cornwalls at the same age, but due to their subsequent intensive growth, the limb bones of the Landraces of 160 days were almost as long as the corresponding bones of the 360-day-old Cornwall pigs.

Richmond and Berg (1972) reported that the growth rate of limb bones except the humerus, in Duroc, Yorkshire and Hampshire breeds decreased after the body weight had reached 90 kg. The femur and the tibia in Landrace hybrids of 100 kg body weight examined by Grøndalen (1974) were almost as long as the same bones in our pigs.

The relative ash content of different limb bones ranged between wide limits in both breeds even at the same age. The highest values were found in the humerus and in the femur. The ash content of these bones in each age group was higher than all the others. The lowest relative bone ash value was measured in the ulna, metacarpus and metatarsus, respectively. The chemical composition of certain limb bones in pigs of the same breed and age was found to be different by Brown (1972), too. This author concluded that the bone metabolism and the calcium absorption and desorption were different related

to either the bones or the age. Field (1974), who studied the data of pigs only in two age groups, also showed that the bone ash per cent for a bone increased with the length of the bone and the age of the pig. He also found the highest ash content in the femur among the bones examined. In Stockland's (1973) study, the bone ash ratio of metacarpus in pigs for slaughter was similar to, while in Doige's study (1975) it was slightly higher, than our values.

The relative mineral content of bones, i.e. the degree and speed of mineralization showed different values, depending on age. The ash content of the humerus, femur and tibia displayed little, if any, change till the age of 3–6 weeks, but showed a downward tendency in the radius, ulna as well as in the metacarpus and metatarsus of Landraces. However, subsequently, the minerals working their way into the bones considerably exceeded in quantity that of the organic matter. The upward tendency of the data in Table II, in accordance with our other examinations, show that the increase of mineral quantity in limb bones of Cornwall pigs had not reached an end by the age of 720 days.

Comparing the two breeds we can state that the mineral content in the Landraces' bones generally exceeded that of the Cornwalls. The difference was especially striking in the case of the radius and the femur.

The data of our examination also showed that neither the metabolism nor the linear growth of the Cornwall and Landrace pigs were the same. During the first days after birth, the bones grew quickly, while the relative bone ash changed almost nothing.

During the period around weaning, the bones grew at a high rate, but in many of the bones the relative ash content was the smallest at the same time. Consequently, at that time the minerals infiltrated the bones slowly, indicating that organic compounds took a greater part in the bone development. We observed an intense growth both in length and ash content of the bones during the period from weaning till the age of 160 days.

The growth in length and ash content of limb bones of Cornwall pigs became slower after this period, but it reserved its upward tendency till day 720, suggesting that it did not stop even at that age. The bones in Landraces reach the size of bones in Cornwalls within a shorter period, indicating a higher degree of mineralization.

In the stock where the examined Landraces of 160 days and 100 kg were chosen from, we observed lameness which can be considered a syndrome of bone weakness. According to the data in Tables I and II, the limb bones of Landraces were almost the same in both size and ash content as those of the one-year-old, healthy Cornwalls not being liable to lameness. Consequently, this type of lameness cannot be attributed either to inadequate growth of bones or to insufficiency of minerals alone.

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РЕЗЮМЕ

ВОЗМОЖНАЯ ЭПИЗООТОЛОГИЧЕСКАЯ РОЛЬ СОБАКИ, ЗАРАЖЕННОЙ Br. suis, В РАСПРОСТРАНЕНИИ СВИНОГО БРУЦЕЛЛЕЗА

Б. КЕРМЕНДИ и ДЬ. НАДЬ

Докладывается о заражении одного свиного половья бруцеллезом, ставшим путем замены поголовья благополучным по этой заразе. На основании эпизоотологического расследования и лабораторных исследований авторы пришли к заключению, что источником заражения уже благополучного поголовья бруцеллезом явилась бруцеллезная собака-фермы, которая была в контакте с неблагополучным поголовьем. После экспериментального заражения при помощи растертых тканей этой, серологически положительно реагирующей, собаки изолирован штамм биотипа 2 Brucella suis, который оказался тождественным с бруцеллезным штаммом, изолированным из мертвым родившиегося поросенка после наступления бруцеллезной вспышки.

АНТИБАКТЕРИАЛЬНОЕ ДЕЙСТВИЕ СИВУЧИХ ЖИРНЫХ КИСЛОТ В ТОЛСТОМ ОТДЕЛЕ КИШЕЧНИКА НА ПРЕДСТАВИТЕЛЕЙ СЕМЕЙСТВА ENTEROBACTERIACEAE

Л. ПРОХАСКА и Ф. БАРОН

В экспериментах in vitro авторами изучалось антибактериальное действие содержимого толстого отдела кишечника здоровых кроликов и свиней, связанное с наличием в нем сивучих жирных кислот. Действие проверялось на штаммах представителей семейства Enterobacteriaceae (Salmonella, Shigella, кишечная палочка, Proteus). Налицевствующие в кишечном содержимом сивучие жирные кислоты при pH 6,0—6,5 и концентрации 70—120 ммоль/кг тормозили мультипликацию бактерий. Этот антибактериальный эффект выше pH 6,8 перестал действовать. Изученные 55 штамма указанных родов бактерий показывали одинаковую чувствительность к антибактериальному действию сивучих жирных кислот.

Интенсивность антибактериального действия содержимого толстых кишок кролика и свиньи при тех же показателях pH не отличались друг от друга. На основании данных изучения содержимого толстого отдела кишечника человека можно заключить, что налицевствующие в нем сивучие жирные кислоты вызывают тоже антибактериальный эффект. В дискуссии обсуждается роль антибактериального эффекта, связанного с наличием в кишечном содержимом сивучих жирных кислот, в патомеханизме энтеральных поражений.

ЗНАЧЕНИЕ ИЗМЕНЕНИЙ В ПЕЧЕНИ В ПАТОГЕНЕЗЕ И ДИАГНОСТИКЕ ЧУМЫ УТОК

П. КАПП, Ф. ВЕТЕШИ и В. ПАЯ

Согласно наблюдениям авторов при острых случаях чумы уток (вирусный энтерит уток, duck plague) развивается острая дистрофия печени. Взаимосвязь между дистрофией печени и возбудителем авторы доказывают морфологическими исследованиями. Наиболее дружную репликацию вируса авторы зарегистрировали в печени молодых уток при перерождении печеночных клеток, сопровождающемся образованием ядерных включений.

Указывается на то, что у большого процента животных, заболевших острой чумой уток, наступают изменения в печени, поэтому при диагносцировании болезни целесообразно попытаться выявить наличие вируса при помощи электронного микроскопа и изолировать вирус.

На основании морфологического изучения печени уток в более поздние сроки времени после инфекции авторы подчеркивают, что — в разрез с настоящими утверждениями — чума уток является не только острого течения болезнью, ибо в молодняке или взрослых уток она проявляется в хронической форме. В таких случаях в погибших животных налицевствует полуострый или хронический интерстициальный гепатит, который в поголовьях вызывает только спорадическую гибель среди животных.

Диагносцирование хронической формы чумы уток авторы считают очень важным в силу того факта, что в данном случае между налицевствующим возбудителем и защитным механизмом организма возникает относительное равновесие и в поголовье могут остаться вирусоносители.

ВОСПРИИМЧИВОСТЬ ВОДОПЛАВАЮЩЕЙ ДОМАШНЕЙ ПТИЦЫ К ВИРУСУ ЧУМЫ ПТИЦ И ЕЕ РОЛЬ В РАСПРОСТРАНЕНИИ ЗАРАЗЫ

М. А. АЛ ИМАДИ и Я. ТАНИ

Дневные утята, молодняк и взрослые утки, дальше, разного возраста гуси заражались через носовую слизистую и конъюнктиву вакцинными вырусными штаммами — лентогенным (Ла Сота), мезогенным (Хертфордшайр) и велогенным уличным — при разбавлении титра 10^6 и 10^2 ID_{50}/мл ради определения их восприимчивости к вирусу чумы птиц (ВЧП) и их роли в распространении заразы. Зараженные животные изучались потом вирусодиагностическими методами.

Обнаружено, что все возрастные группы данных животных восприимчивы к всем изученным штаммам ВЧП, но их восприимчивость незначительна. Гуси оказались более восприимчивыми, чем утки. Восприимчивость животных с возрастом ослаблялась. Зато серологические ответы, полученные пробой торможения гемагглютинации, с возрастом становились более выразительными.

Вакцинные вирусы размножались только близ места внедрения, то есть в глотке и ее непосредственной окрестности и короткое время выделялись слизью при низком инфекционном титре.

Велогенные вирусные штаммы у водоплавающих птиц вызывали виремию и они выделяли вирус со всеми выделениями в течение 8 дней. С возрастом частота выделения вируса и его концентрация постепенно уменьшались. От большей концентрации вируса дневные гусята всегда заболели после инкубационного времени 3—8 дней и к 1-у—5-у дню болезни погибали. У дневных утят, утиного молодняка и взрослых уток и гусей болезнь проходила в невидимой форме и о ее наличии удалось убедиться только вирусдиагностическими методами.

Поскольку для заражиния водоплавающей птицы ВЧП нужны большие количества вируса, ее заражение им возможно только при тесном контакте с зараженными курами. Из больных или зараженных животных выделяются только виремию вызывающие велогенные штаммы наружу довольно длительное время и в надлежащей концентрации, таким образом они могут быть опасными для более восприимчивых кур.

Нет доказательства о том, что водоплавающая птица может быть длительное время вирусносителем.

Ее роль в распространении ВЧП может быть опасной только в приусадебных условиях или в природе, но эту опасность карантинными мероприятиями во время эпизоотии можно до минимума снизить.

СРАВНЕНИЕ ГЕМАГГЛЮТИНАЦИОННОЙ АКТИВНОСТИ И ИММУНОГЕННОГО ДЕЙСТВИЯ ВИРУСНЫХ ШТАММОВ БЫЧЬЯ ПАРАИМФЛЮЭНЦА-3

Б. КЁВЕШ, Ш. БЕЛАК и М. РУШВАИ

При изучении вирусных штаммов параинфлюэнца-3 (ПИ-3), изолированных из 6—8-недельных и 5—12-месячных телят, болеющих разными формами болезни дыхательных путей, авторами обнаружено, что гемагглютинационная (ГА) активность изолятов такого же инфекционного титра существенно отклонялась друг от друга и этого свойства они не теряли и после ряда пассажей.

Вирусные штаммы ПИ-3 сильной ГА активности подвергались тяжелым физическим и химическим воздействиям при чем констатировано, что некоторые среди них, как то, воздействие температурой 50°С в течение 120 минут или обработка 0,1%-ным трипсином в

течение 30 минут равным образом уничтожают их инфективность и ГА способность. Другие способы воздействия, как то, выдержка при температуре 50°С в течение 50 минут или обработка 0,25%-ным трипсином в течение 30 минут при неповреждении их инфективности полностью уничтожают ГА способность этих вирусных штаммов. Другие воздействия, как например обработка формалином или бета-пропиолактоном уничтожают инфекивность вируса и в разной мере повреждают гемагглютинины. В исследованиях авторов этиленимин инфективность ПИ-3 полностью уничтожил, но не действовал на ГА активность.

Между иммуногенным действием и ГА активностью как оригинально высокой, так и оригинально низкой ГА активности штаммов, дальше, разным образом поврежденных таковых имеется положительная взаимосвязь.

В два раза привитых кроликах инактивированным этиленимином вирусом ПИ-3 обнаружено, что гемагглютинацию тормозящие антитела появляются уже после первой прививки, а заметное повышение вирус-нейтрализирующих таковых наблюдается только после второй прививки.

ЭКСПЕРИМЕНТЫ ПО ИММУНИЗАЦИИ ИНАКТИВИРОВАННЫМ ВИРУСОМ ПАРАИНФЛЮЭНЗА-3

Б. КЁВЕШ, Ш. БЕЛАК, М. РУШВАИ и Р. ГЛАВИЧ

Авторами изучались особенности иммунологического ответа, вызванного изолированным из дыхательных путей больных телят сильной гемагглютинационной (ГА) активности вирусным штаммом параинфлюэнза-3 (ПИ-3), инактивированным этиленимином. Обнаружено, что уровень гемагглютинацию тормозящих (ГАТ) и вирунейтрализирующих (ВН) антител у дважды — с промежутками времени 7, 10, 14 и 21 дней соответственно — привитых и в те же сроки изученных животных сильно отличался друг от друга. Касательно антител ГАТ зарегистрировано математически достоверное заметное повышение титра по сравнению с основным таковым во всех прививочных программах. ВН антитела появились после прививки и математически достоверное повышение их титра наблюдалось только после второй таковой, когда они осуществлялись с промежутком времени в 14 дней. При сравнении прививочных программ к 14-у и 28-у дням после второй прививки в отношении ВН антител обнаружено математически достоверное превосходство той, где вторую прививку осуществляли 14 дней после первой, тогда как по наличию ГАТ антител существенной разницы не было между разными прививочными программами.

После иммунизации ягнят вирусом ПИ-3 авторы изучали иммунологический ответ. В срок формирования кривой антител вниз животных перезаражали вирусом ПИ-3. Неиммунизировавшиеся животные клинически заболели, выделяли вирус и у них наступили патогистологические изменения. У иммунизировавшихся животных клинических признаков заболевания не наблюдали, вирус они не выделяли, но незначительные патогистологические изменения у них имелись. Территории ответственных за целлюлярный иммунитет клеток показывали в лимфатической системе сильную пролиферацию.

Результаты исследований показывают, что вирусом с высокой ГА активностью, инактивированным этиленилином, можно вызвать иммунитет против вирусной заразы ПИ-3.

СЕРОЛОГИЧЕСКОЕ ИЗУЧЕНИЕ НАЛИЧИЯ АНТИТЕЛ ПРОТИВ РОТАВИРУСОВ В СВИНОПОГОЛОВЬЯХ ВЕНГРИИ ПУТЕМ ИММУНОЭЛЕКТРОФОРЕЗА С ПРОТИВОТОКОМ

Э. МОЧАРИ, ИРЕН ХОРВАТ и Э. КУДРОН

552 образца крови, собранные из 44 крупных свиноферм 13 областей Венгрии, изучены на наличие специфических для ротавирусов антител путем иммуноэлектрофореза с противотоком (ИЭФПТ). Среди технических условий, сказывающихся на надежности и специфичности пробы качество антигена и тип агарозы являются определяющими.

Антиген приготовлен из испражнений искусственно зараженного теленка путем центрифугирования, фильтрации и ультрацентрифугирования. Его титры колебались в пределах 1 : 16 – 1 : 128. ИЭФПТ осуществлялся на микроскопических предметных стеклах, покрытых 4,5 мл-ами желя 1%-ной агарозы А (Фармация) в барбитальном буфере при pH 6,8. Электрофорез проходил при скорости 7 V/см в течение 90 минут.

Среди изученных ферм 41 (91,2%) оказалась неблагополучной по ротавирусу. К ротавирусу специфические антитела обнаружены в 167 образцах крови (31,2%). Количество положительно реагирующих свиней на изученных фермах колебалось в пределах 6,7—100%. Наличие антител в 4-х, 6-и, 8-месячных и годовалых и старше свиней равнялось 26, 50,6 33,3 и 25,2%-ам соответственно. Результаты исследований показали, что большинство свиней заражается ротавирусом в возрасте после отбивки.

КОЛИЧЕСТВЕННОЕ ОПРЕДЕЛЕНИЕ НЕКОТОРЫХ ФУЗАРИЙНЫХ ТОКСИНОВ ГАЗОВО-ХРОМАТОГРАФИЧЕСКИМ МЕТОДОМ

А. ВАНИ, А. БАТА и ЛАСТИЧ Р.

Разработан метод симультанного определения пяти фузарийных микотоксинов: деоксиниваленола, диацетоксицирпенола, НТ-2, Т-2 и зеараленона.

Образцы зерновых кормов экстрагировались уксуснокислым этилем, потем смесью метанола с водой соотношения 6 : 4. Экстракт очищался при помощи Киселжель 60 колончатой хроматографией. Очищенные при помощи Киселжель 60 колончатой хроматографией. Очищенные образцы реактивировались и, 0-бис (триметисилил)-трифторацетамидом. Производные силилэфира образцов анализировались при помощи открытой тубулярной колонки SE 52, нанесением на стенку. Чистые токсины смешивались с образцами зерновых кормов и осуществлялись идентификация токсинов и тесты стандартной девиации. 70—80%-ов токсина удавалось выявить и относительная стандартная девиация колебалась в пределах 10—18%-ов. Разработанный метод был проверен на конкретных образцах.

ДЕЙСТВИЕ ВЫЗВАННОГО В МАТЕРИНСКОМ ОРГАНИЗМЕ АЦИДОЗА НА БАЛАНС КИСЛОТА-ЩЕЛОЧЬ У НОВОРОЖДЕННОГО ТЕЛЕНКА

О. СЕНЦИ, Ф. КУТАШ и Я. ХАРАСТИ

Непосредственно перед родом у семи нетелей и коров авторами вызван метаболический ацидоз добавлением к корму тростникового сахара. Две стельные нетели служили контролем. Целью исследований явилось изучение того, что насколько действует экспериментальный ацидоз матери на баланс кислота-щелочь и жизненность плода и новорожденного теленка.

Согласно параметрам крови и мочи острый ацидоз матери, продолжающийся одни сутки не сказывается отрицательно на балансе кислота-щелочь новорожденного теленка; он не отличался от такового контрольных телят. Зато продолжающийся 4—7 дней и постепенно усиливающийся ацидоз матери обусловливает рождение ацидозных телят; в обратном случае у последних несколько часов спустя развивается ацидоз. Два ацидозных теленка после рождения скоро погибли.

ИЗУЧЕНИЕ ВЗАИМОСВЯЗИ МЕЖДУ НОВОЙ БАЛЛОВОЙ СИСТЕМОЙ ОПРЕДЕЛЕНИЯ СОСТОЯНИЯ НОВОРОЖДЕННЫХ ТЕЛЯТ И ИХ БАЛАНСОМ КИСЛОТА-ЩЕЛОЧЬ

О. СЕНЦИ

Аппликацией медицинского опыта диагностики состояния младенцев автор рекомендует новый, простой и легко усвоимый балльный метод диагностики состояния новорожденных телят, который сравнивается с балансом кислота-щелочь.

При помощи данного балльного метода в согласии с балансом кислота-щелочь без лабораторного измерения можно определить состояние новорожденного теленка и тем самым заблаговременно можно приступить к необходимой терапии животного.

ПЕРИНАТАЛЬНАЯ СМЕРТНОСТЬ НА ФЕРМАХ КОРОВ ГОСУДАРСТВЕННЫХ ХОЗЯЙСТВ

О. СЕНЦИ и М. КИШШ

Авторами проанализированны данные 163-х ферм коров 59-и государственных хозяйств за 1978—79 годы. Рождение мертвых телят группировали следующим образом: по породам (табл. 1,;2,), в разбивке по годам и месяцам (табл. 3), по коровам и нетелям

(табл. 4), по полам (табл. 5), согласно количеству молока (табл. 6), по размеру фермы (табл. 7), по количеству спецработников по отелу (табл. 8), по образованию дежурящих в родильном помещении ночью и в выходные дни (табл. 9), согласно структуре родильного помещения и технологии отела (табл. 10), по содержанию в период сухостоя (табл. 11).

ИЗМЕНЕНИЯ НЕКОТОРЫХ ЛИПИДНЫХ СОСТАВНЫХ ЧАСТЕЙ В СЫВОРОТКЕ МОЛОЧНЫХ КОРОВ В ПЕРИОД ОТЕЛА

Ф. ХУШВЕТ, Ф. КАРШАИ и Т. ГААЛ

Авторами обнаружено, что в период отела содержание всего липида и всего холестерола в сыворотке молочных коров математически достоверно существенно меньше, чем до или после данного времени. Уровень всего липида понижается в среднем на 35%.

Количество свободных жирных кислот во время отела повысилось в три раза раньше определенного показателя. Содержание свободных жирных кислот во всем липиде сыворотки во время отела равнялось 4,5%-ам, тогда как раньше и позже оно было только 1,1—1,2%.

Содержание всего липида в печени в период отела было в полтора раза больше среднего показателя. Два месяца после отела уровень липида в печени понизился до его показателя времени сухостоя.

В липиде как сыворотки так и печени равным образом определено по 12 жирных кислот. Среди них C16 : 0, C16 : 1, C18 : 0, C18 : 1, и C20 : 4 показывало математически достоверные отклонения, а C12 : 0, C14 : 0, C17 : 0, C18 : 3, C20:0 и C22 : 0 математически недостоверные таковые.

В липиде сыворотки соотношение C18 : 0, C18 : 1 и C18 : 2 равнялось 85—90%-ам. В липиде печени соотношение C16 : 0, C18 : 0 и C18 : 1 равнялось 70—80%-ам.

Вопреки количественным отклонениям во формировании уровня жирных кислот в сывороточном и печеночном липидах обнаружена многосторонняя сходность. Одновременно полученные данные показали, что в этих двух веществах характерные изменения некоторых жирных кислот быстро и консеквентно следовали друг за другом. Однако разницей нужно считать тот факт, что процентное соотношение жирных кислот в липиде печени показывало больший диапазон колебания, чем в сывороточном липиде. В период отела равным образом как в сывороточном, так и печеночном липидах наблюдали повышение. уровня C16 : 0, C16 : 1, и C18 : 1 и понижение такового C14 : 0, C18 : 0, C18 : 2 и C20 : 4.

На основании отклонений параметров обмена сывороточного и печеночного липидов можно заключить, что в период отела у здоровых коров в обмене жиров наступают существенные, но физиологические изменения, которые наблюдаются при голодании или кетозе. Такое положение может затруднить дифференциацию нормальных и патологических явлений.

ГИСТОЛОГИЧЕСКОЕ ИЗУЧЕНИЕ ФОСФАТАЗ И НЕСПЕЦИФИЧЕСКИХ ЭСТЕРАЗ В ГИПОФИЗЕ У КОЗЫ И БУЙВОЛА

Р. П. САЙГАЛ, Б. С. НАНДА и С. К. НАГПАЛ

Криостатические срезы гипофиза козы и буйвола подвергались разным гисто энзиматическим исследованиям для выявления в них щелочной фосфатазы, кислой фосфатазы, 5-нуклеотидазы, аденозин трифосфатазы, глюкоза-6-фосфатазы и неспецифической эстеразы.

Интенсивность большинства энзиматических активностей существенно отличались в разных клеточных группах дистальной части гипофиза у обоих видов животных. Энзиматические реакции были те же в дистальной части гипофиза у обоих видов животных за исключением щелочной фосфатазы, которая у козы была ограничена к эндотелию капилляров, тогда как она у буйволов налицевствовала и внутрицеллюлярно. Активностей аденозин трифосфатазы и 5-нуклеотазы не наблюдали в дистальной части гипофиза буйвола.

Все изучавшиеся энзимы обнаруживались в разной концентрации в клетках средней части гипофиза обоих животных за исключением аденозин трифосфатазы и 5-нуклеотидазы, которые у буйвола и в этой части гипофиза отсутствовали.

В нервной части гипофиза реакции энзимов щелочной фосфатазы и аденозин трифосфатазы у козы наблюдались только в эндотелии, тогда как у буйвола здесь наблюдали толь-

ко реакцию щелочной фосфатазы; реакции 5-нуклеотидазы в этой части органа не обнаружено ни у одного вида изучавшихся животных. Наиболее сильную реакцию в питуицитах показывала кислая фосфатаза, а вдоль нервных волокон — неспецифическая эстераза.

ПОПЫТКА ЭКСПЕРИМЕНТАЛЬНОГО ИЗУЧЕНИЯ ПАТОГЕНЕЗА ГЕНЕРАЛИЗИРОВАННОГО ТОКСОПЛАЗМОЗА У СОБАКИ

М. ДОБОШ-КОВАЧ и И. ВАРГА

Серологически раньше неизученных 48 одно-трехмесячных щенков авторы заразили через рот цистами или ооцистами Toxoplasma gondii. Ради получения иммунодепрессии часть щенков обработали бетаметазоном (Бетсолан инъ. Глаксовет; см. табл. 1). У одной третьи обработанных бетаметазоном щенков после инвазии цистами или ооцистами возник генерализированный токсоплазмоз и они погибли. Генерализированный токсоплазмоз и, следовательно, гибель экспериментальных животных наступили между 2-й и 3-й неделей после инвазии. У одной части животных токсоплазмоз не генерализировался и примерно у половины щенков не получено заражения. Среди необработанных бетаметазоном животных генерализированный токсоплазмоз обнаруживался только случайно и частота негенерализированного токсоплазмоза была существенно реже, чем в обработанной группе (см. табл. 2). Данные экспериментов являются доказательством того, что у собаки генерализированный токсоплазмоз развивается только вследствие понижения иммунологической реагируемости организма.

ИЗУЧЕНИЕ ТЕРАТОЛОГИЧЕСКОГО ДЕЙСТВИЯ МЕТИЛПАРАТИОН 18 ВП И ВОФАТОКС 50 ЕЦ НА ЭМБРИОНАХ НЕМОГО ПЕРЕПЕЛА И ФАЗАНА С ОСОБЫМ ВНИМАНИЕМ НА КОСТНЫЙ СКЕЛЕТ И МЫШЦЫ

Л. ВАРНАДЬ, РОЖА ИМРЕ, Т. ФАНЧИ и А. ХАДХАЗИ

Авторами изучено тератологическое действие инсектицидов пылеобразного метилпаратиона 18 ВП и взвесь образующего вофатокс 50 ЕЦ на эмбрионах немого перепела и фазана с особым вниманием на костный скелет и мышцы.

Обработка яиц осуществлялась 0,05, 0,5, и 5,0%-ными суспенсиями метилпаратиона 18 ВП и 0,02, 02, и 2,0%-ными взвесями вофатокс 50 ЕЦ. Эти вещества вводились в воздушную камеру яиц в количестве 0,5 и 0,1 мл соответственно, в случае перепелов к 9-у, а в случае фазанов — к 12-у дню инкубации. Изучение эмбрионов осуществлялось к 14-у и 23-у дню инкубации соответственно.

В костях эмбрионов с лордоксолиозом (наичаще обнаруженная ненормальност развития) применявшимися методами изучения изменений не обнаружено. Шейные позвонки макро- и микроскопически были нормальными. В шейной мускулатуре эмбрионов, обработанных высокими дозами вофатокс 50 ЕЦ, наблюдалась гистологическая картина, напоминающая атрофию или местами гипоплазию.

На основании своих наблюдений авторы обращают внимание на то, что обработка птичьих эмбрионов фосфорорганическими соединениями, особенно при лордосколиозе, может тормозить иннервацию. Вследствие этого состояния может наступить атрофия шейных мышц и, тем самым, вызвать ненормальную постановку шеи и головы.

При хорошей практике защиты растений с экотоксической точки зрения изученные препараты авторы не считают опасными, поскольку структура яйцевой оболочки обеспечивает надлежащую защиту эмбриона от факторов окружающей среды, в том числе от изученных инсектицидов в указанных концентрациях.

ДАННЫЕ К АНАТОМИИ СУХОЖИЛИЯ ДВУХГЛАВОГО МУСКУЛА ПЛЕЧА ЛОШАДИ И LACERTUS FIBROSUS

Ф. ДЮРЮ и Й. ЗАЙЕР

На трупах 4-х плодов, 5-и новорожденных жеребят и 91 взрослой лошади (возраста 1—23 лет) авторами изучено сухожилие, проходящее в брюшке двухглавого мускула плеча лошади и lacertus fibrosus. На двух оперированных лошадях кроме этого изучались перебои в функции конечности после пересечения сухжилия и lacertus fibrosus.

Обнаружено, что сухожилие в брюшке двухглавого мускула плеча лошади это не уплотнение фасции плеча, его нельзя считать межмускульной прослойкой, а является непосредственным продолжением его начального сухожилия. Проходящее в брюшке мускула сухожилие и сам мускул единым сухожилием прикрепляется к шероховатости лучевой кости. В брюшке мускула сухожилие в латеральную сторону скручено, что обеспечивает лучший пружинный эффект и повышает силу напряжения. На основании возникновения, прохождения и функции авторы рекомендуют его называть "tendo scapuloradialis".

При помощи операции доказано, что после пересечения данного сухожилия двухглавый мускул плеча остается непосврежденным, его волокна свою функцию без отказа выполняют, хотя синхронная функция плечевого и локтевого суставов немножко ухудшается. При движении наблюдаются перебои в продвижении конечности вперед.

После полного пересечения двухглавого мускула плеча, без пересечения lacertus fibrosus локтевой сустав можно настолько разогнуть, что при натяжении конечности назад копыто можно приблизить к колену. Это явление нужно считать диагностического значения.

Lacertus fibrosus нельзя считать единственным продолжением проходяшего в брюшке сухожилия двухглавого мускула плеча и в новейшее время в литературе фигурирующей латеральной ветвью сухожилия. Lacertus fibrosus обладает центральной и двумя боковыми пластинками. Только его центральная часть является сухожильного происхождения. Его боковые пластинки являются производными фасции, которые у взрослых дошадей представляют собой 4/5 толщины lacertus fibrosus.

При пересечении lacartus fibrosus при движении наблюдается незначительная хромота типа нагрузки.

РАЗВИТИЕ КОСТЕЙ КОНЕЧНОСТЕЙ У СВИНЬИ

М. СИЛАДИ, Г. КЁКЕНЬ и А. Б. КОВАЧ

Авторами определялись и сравнивались длина и содержание золы костей конечностей (плечевой, лучевой, локтевой, пястной IV, бедренной, большой берцовой, плюсневой IV) свиней разного возраста пород корнвол и низменная гибридная.

Обнаружено, что длина костей конечностей у породы корнвол примерно до 80—100-дневного возраста росла быстрее, чем у свиней низменной породы, но после этого кости конечностей свиней низменной породы становились существенно длиннее.

Содержание золы костей соразмерно возрасту почти одинаковым образом росло у обеих пород за исключением периода около отбивки, когда во всех костях по сравнению с раньшими показателями оно понизилось.

Примерно у 30%-ов свиней породы низменный гибрид, возраста 160 дней и живого веса 100 кг наблюдали хромоту, принадлежащую в понятие синдром слабости костей. Длина и содержание золы в костях этих свиней практически не отличались от тех же показателей одногодичных, здоровых несклонных к хромоте животных породы корнвол. Согласно авторам возникновение хромоты нельзя объяснить ненормальным ростом длины костей или недостатком минеральных веществ.

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SECONDARY RENAL AMYLOIDOSIS AND ITS CONSEQUENCES IN THE DOG

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The occurrence of five spontaneous cases of renal amyloidosis in the dog is reported on the basis of necropsy findings. Among dogs submitted for necropsy, 2 were diagnosed as having nephrotic syndrome associated with renal amyloidosis, 2 showed thrombosis of the pulmonary artery, pulmonary veins and vena cava caudalis as sequelae to nephrotic syndrome, and 1 had amyloid nephrocirrhosis complicated with prolonged uraemia. In all five cases the diagnosis of secondary renal amyloidosis was confirmed by histopathological methods.

The presumptive pathogenesis of the thrombotic tendency as a complication of the nephrotic syndrome associated with renal amyloidosis is discussed.

The occurrence of different disease entities characterized by regressive changes of glomeruli (glomerulonephrosis) in animals has rarely been reported. Although renal amyloidosis of the dog has long been known, it has only rarely been mentioned in the literature.

Amyloidosis in the dog is considered to be a secondary pathological condition following a primary disease. It occurs at most in 1% of dogs submitted for necropsy (Osborne et al., 1968). Recently it has been reported to occur in dogs in association with tuberculosis (Dahme and Weiss, 1978; Eikmeir and Moegle, 1958), systemic lupus erythematosus (Lewis and Hathaway, 1967), blastomycosis (Sherwood et al., 1967), cyclic neutropenia (gray collie syndrome) (Cheville, 1968), pleuritis caused by *Nocardia asteroides*, coccidiomycosis, chronic purulent metritis, purulent arthritis complicated with osteomyelitis, lymphosarcoma, pyoderma (Slauson et al., 1970), pyoderma and ulcerative perianal adenoma, purulent prostatitis and phlegmone (Osborne et al., 1969). Amyloid deposition in the dog frequently occurs in a generalized form, affecting several organs, while in other cases it is restricted to the kidney (Osborne et al., 1968; Watson, 1971).

In cases when renal lesions are in the foreground or only the kidneys are affected, a unique disease entity, nephrotic syndrome, may develop in the dog. Nephrotic syndrome has already been reported by several authors (Lapras, 1979; Osborne et al., 1968, 1969; Slauson et al., 1970; Watson, 1971). Thrombosis of certain blood vessels has also been observed in connection with renal amyloidosis and with the so-called nephrotic syndrome associated with amyloidosis (Slauson and Gribble, 1971).

Materials and methods

During the last few years, renal amyloidosis was diagnosed in 5 dogs of those submitted for necropsy, viz. a 6-year-old smooth-haired male Hungarian retriever (Case 1), a 9-year-old female Polish sheepdog (Case 2), a 5-year-old black male Puli (Case 3), a 13-year-old female mongrel (Case 4) and a 9-year-old female skye terrier (Case 5). It should be noted that none of the 5 animals was subjected to previous clinical examination, since dogs Nos 1 and 2 were killed due to their progressed state of illness at their owners' request, without a thorough previous examination, while dogs Nos 3, 4 and 5 had died before their owners could have consulted a veterinarian.

Organs of dogs subjected to necropsy were fixed in a neutral formaldehyde solution of 2.66 mol/l (8%) and embedded in paraffin wax. In preparing the slides for histopathology, in addition to the usual haemalaun-eosin staining, the Congo red and gentian violet staining and the iodine reaction were used to detect amyloid. To render identification of amyloid possible, the sections stained with Congo red were covered with gum arabic and the preparations were subjected to polarization-optical studies (Romhányi, 1971 and 1979).

For determining the nature (primary or secondary character) of amyloid, the deparaffinized sections were pretreated with a specific mixture of concentrated formic acid and hydrogen peroxide (so-called performic acid), as recommended by Romhányi (1979), then stained with Congo red, covered with gum arabic, and examined in a polarization microscope.

For further studies on the properties of amyloid, deparaffinized sections were pretreated with a 1 : 1 mixture of 0.25% potassium permanganate and 0.3% sulphuric acid, according to the method of Romhányi (1972 and 1979). This was followed by the digestion of the pretreated sections with trypsin for 2 and 24 h.

For the demonstration of amyloid, the macroscopic iodine reaction was also performed on the cut surface of kidneys of dogs, with Lugol's solution acidified with sulphuric acid.

For electron microscopic examinations, kidney samples from dogs were fixed also in an osmium tetroxide solution prepared with Caulfield's buffer. The material was embedded in Durcupan. Semi-thin sections prepared from the embedded material were stained with Jones's staining combined with Congo red. Ultrathin sections were examined by transmission electron microscopy.

Urine samples taken from the urinary bladder of dogs submitted for necropsy were subjected to the sulphosalicylic acid test for protein demonstration. In addition, in urine samples collected from urinary bladders of dogs Nos 3, 4 and 5, specific gravity determinations and a quantitative determina-

tion of total protein and albumin content were performed. Total protein content was determined by the biuret reaction (Weichselbaum, 1946), while albumin was demonstrated by the bromocresol green reaction (Gustafson, 1976). Determinations were made in a Satellite NK-230 automatic one-channel clinicochemical analyser (Székely and Bartalits, 1979).

Results

Necropsy findings

Dogs Nos 1 and 2 were in a severely emaciated condition before killed. There was marked subcutaneous oedema along the sternum in both animals, and in the male dog also around the prepuce. The abdominal cavity contained approximately 3 l and 1.5 l of transparent serous exudate in Cases 1 and 2, respectively. There were 100 ml and 200 ml quantities of serous exudate also in the thoracic cavities of dogs Nos 1 and 2, respectively. The liver showed congestive hyperaemia, the lungs acute oedema, while in the heart a general simple dilatation was observed.

The kidneys of carcases were pale, moderately enlarged and of increased consistency; on their cut surfaces the glomeruli were clearly visible, and the fine structure of the cortex of kidneys was indistinct. There were no pathological lesions in other organs.

The sulphosalicylic acid test demonstrated the presence of large quantities of protein in urine samples obtained from the urinary bladder of both dogs.

Dogs Nos 3 and 4 were in an average body condition prior to their death. In dogs Nos 3 and 4, respectively, the abdominal cavity contained

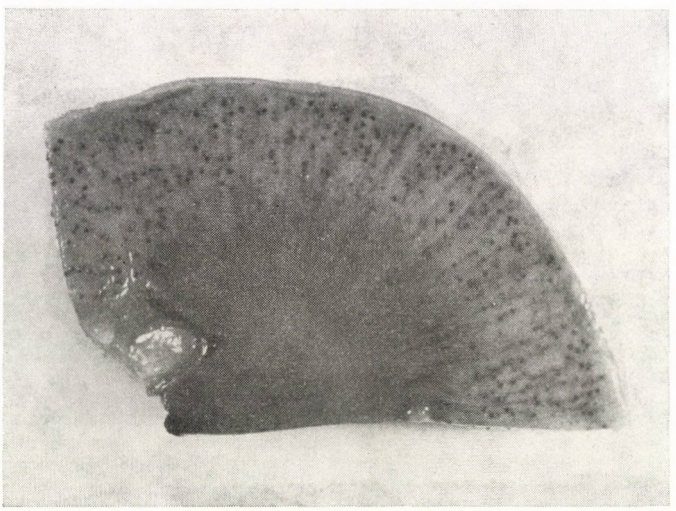

Fig. 1. Macroscopic iodine reaction on the cut surface of the kidney. Amyloid-containing glomeruli stain deeper. (Magnified)

approximately 1.5 l and 300 ml, while the thoracic cavity approximately 300 and 100 ml of straw-coloured serous exudate. The renal capsules were tight and the kidneys pale, swollen and of increased consistency. Glomeruli were clearly visible on the cut surface, and the fine structure of the renal cortex was indistinct. The consistency of the cortex was friable. In the macroscopic iodine reaction performed with Lugol's solution acidified with sulphuric acid, glomeruli showed a bluish-brown colour characteristic of amyloid (Fig. 1).

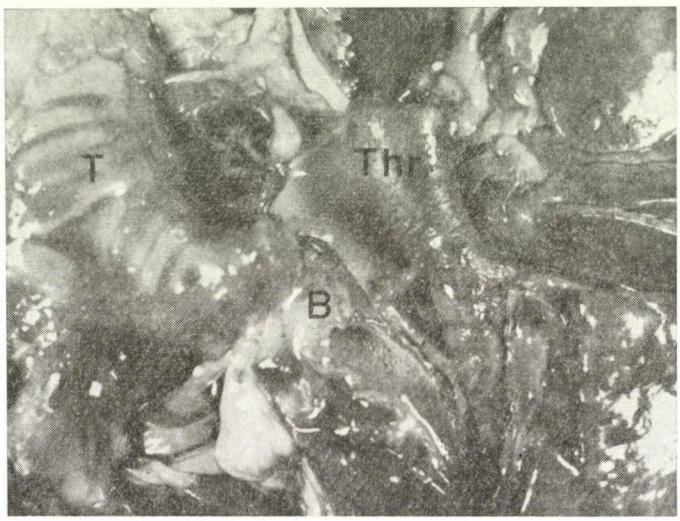

Fig. 2. Mixed (stratified) thrombus obliterating the pulmonary artery (the wall of the artery is opened). T = trachea, B = bronchus, Thr = thrombus. (Magnified)

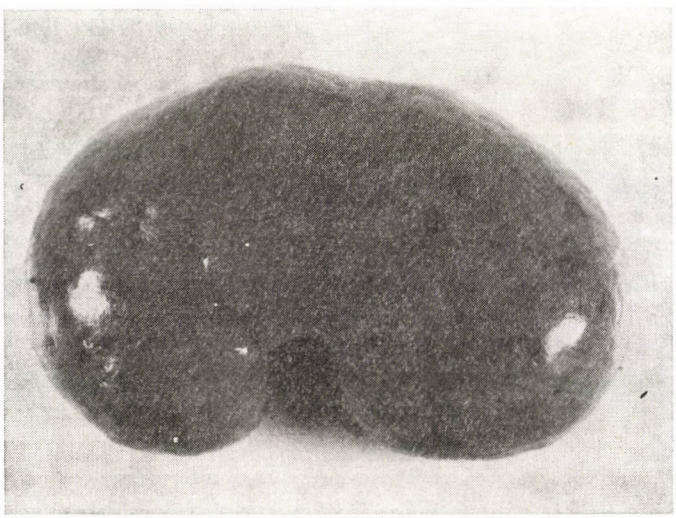

Fig. 3. Amyloidic contracted kidney of greyish-white network pattern. (Magnified)

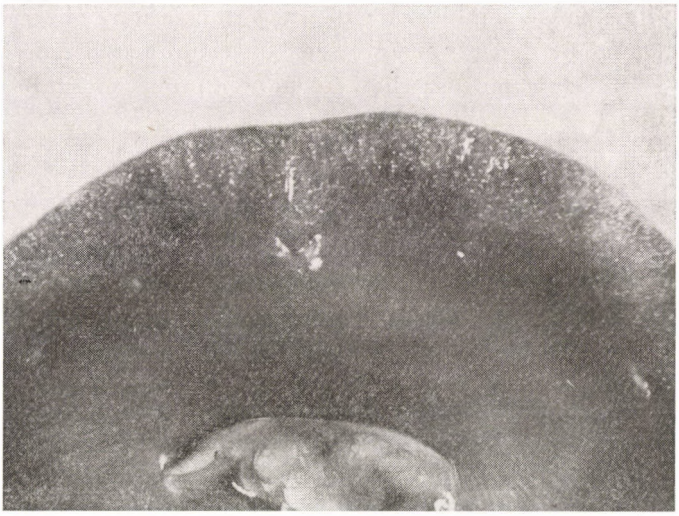

Fig. 4. Glomerular amyloidosis. Clearly visible glomeruli on the cut surface of the renal cortex. (Magnified)

The lungs were hyperaemic and oedematous in both cases. In dog No. 3, the trunk of the pulmonary artery and the pulmonary artery itself in both lung halves (from its origin to the small arterial branches 1 mm in diameter) was filled with a fresh thrombus of mixed character (Fig. 2). In dog No. 4, the lumina of the pulmonary veins and the vena cava caudalis contained obstructing thrombi. These were not adhered to the wall of the vessels. Blood vessels of other organs were not found to contain thrombi. The liver showed severe acute congestive hyperaemia in both cases. No important lesions were found in other organs.

The total protein contents of urine samples collected from the urinary bladder of the two canine carcases were 9.3 and 38.0 g/l, respectively, and their albumin concentrations were 4.2 and 3.7 g/l, respectively. The respective urinary specific gravity values were 1.012 and 1.026.

At necropsy, the body condition of dog No. 5 (which had also died) was poorer than average. Both kidneys were slightly reduced in size, pale, firm and of slightly uneven surface, and their substances had a greyish-white mottled appearance (Fig. 3). On the cut surface of the kidneys the glomeruli were clearly visible in the thinned-down cortex (Fig. 4). The structure of the kidneys was indistinct and their substance was harder to tear than usual. In the macroscopic reaction performed with Lugol's solution and diluted sulphuric acid, the glomeruli showed a discoloration characteristic of amyloid.

In addition to renal lesions, myocardial hypertrophy of the left half of the heart, chronic endocarditis associated with connective tissue proliferation in the atrioventricular valves of both heart halves, endometrial cystic hyper-

plasia and — as a consequence — incipient pyometra were observed. Uraemic erosions and incipient ulceration were seen on the mucosal membrane of the oral cavity. The gastric mucosa showed the symptoms of acute catarrh and had a pungent odour characteristic of ammonia.

Total protein and albumin concentrations of the urine collected from the urinary bladder of the carcase were 4.6 g/l and 2.9 g/l, respectively. The urine had a specific gravity of 1.009.

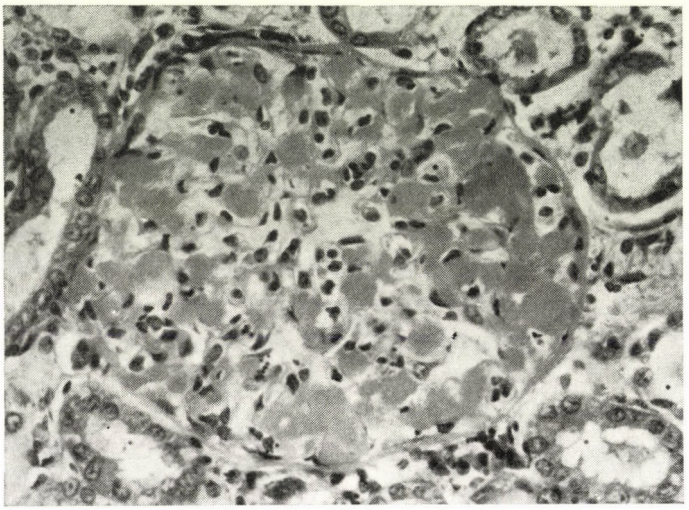

Fig. 5. Homogeneously-staining amyloid deposition in the walls of the glomerular blood vessels. The glomerular structure is disintegrated. Congo red staining, approx. ×420

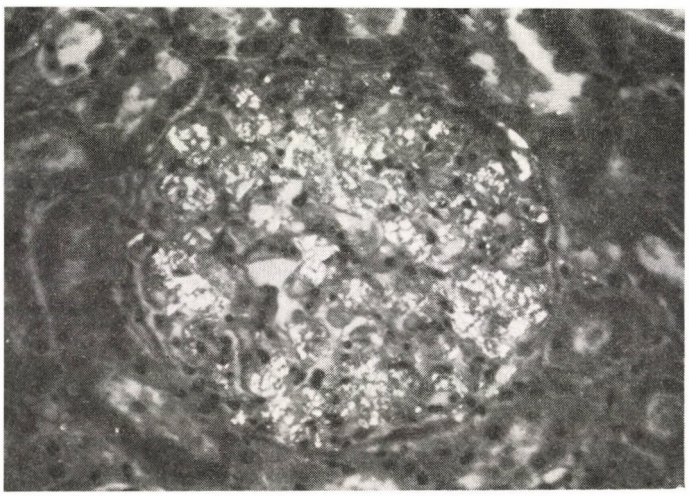

Fig. 6. Birefringent amyloid in the glomerulus. Congo red staining, section covered with gum arabic. Polarization optical photograph, approx. ×330

Histopathological examination

Histopathological examination performed by light microscopy revealed disintegration of glomerular structure in all five cases. In the relatively cell-deficient glomeruli, the capillary loops had an unevenly thick appearance due to the deposition of a homogeneously-staining substance (Fig. 5). The deposited material showed staining reactions characteristic of amyloid: orange-red with Congo red, brown with Lugol's solution, red with gentian violet. When examining the sections stained with Congo red and covered with gum arabic in polarized light, birefringence of greenish-yellow polarization colour was observed (Fig. 6).

It was apparent in both the conventional light-microscopic and the semi-thin sections that amyloid deposited primarily between the basement membrane of glomerular capillaries and the endothelial cells lining the capillaries (subendothelially), producing uneven deposits. Amyloid deposits caused marked stenosis of certain capillaries (Fig. 7). In some places, amyloid deposited not only subendothelially, but also between the podocytes (visceral epithelial cells) covering the capillary loops and the basement membranes of capillaries (subepithelially). The amyloid deposited subepithelially consisted of radial bundles located in the interspaces between the projections of podocytes (Fig. 8). In the basic substance of the mesangium, amyloid was demonstrated neither in the semi-thin sections, nor by electron microscopic examinations.

In dogs Nos 1, 2, 3 and 4, the majority of tubules were found to show accumulation of homogeneously-staining material (hyaline cylinders) in their lumina. In these cases the epithelial cells of the proximal convoluted tubules

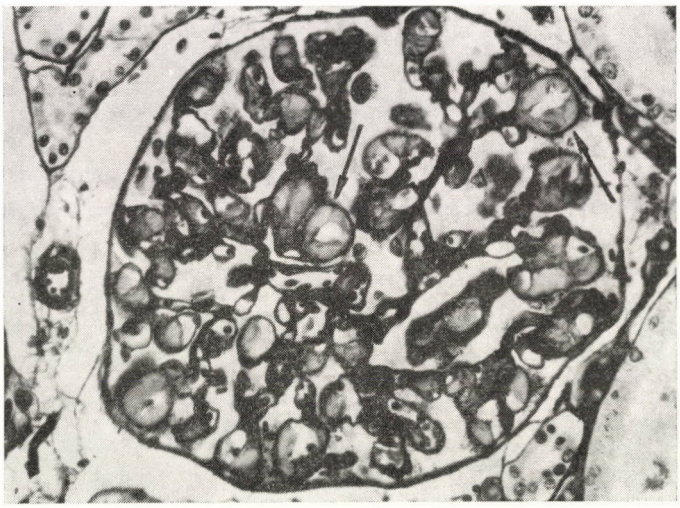

Fig. 7. Stenosis of the lumen of glomerular capillaries (arrow), due to subendothelial deposition of amyloid. Jones's staining, approx. ×420

showed a hyaline degeneration of varying extent associated with karyopycnosis of epithelial cells. The intertubular connective tissue was usually moderately infiltrated with serous exudate.

In the kidneys of dog No. 3, a very mild, focal, subacute inflammation resulting from ascending urinary infection was observed.

In the kidneys of dog No. 5, in addition to glomerular lesions, many tubules exhibited signs of degeneration and atrophy, or complete destruction

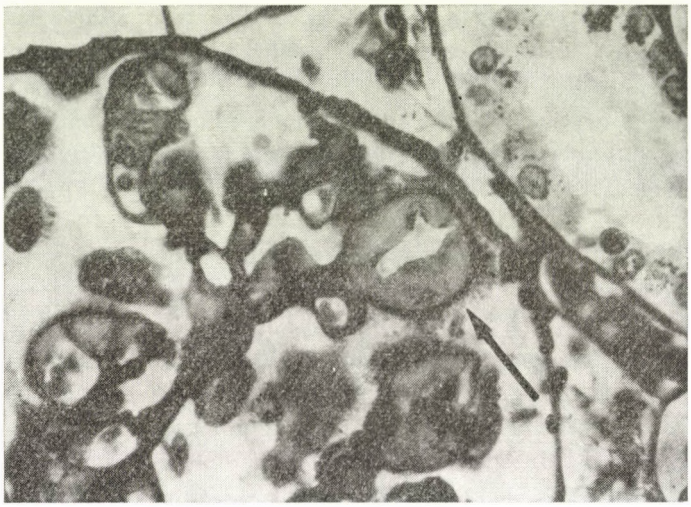

Fig. 8. Narrowed lumen of blood vessel due to subendothelial deposition of amyloid (arrow). Amyloid forms radial bundles on the outer surface of capillaries. Jones's staining, approx. ×850

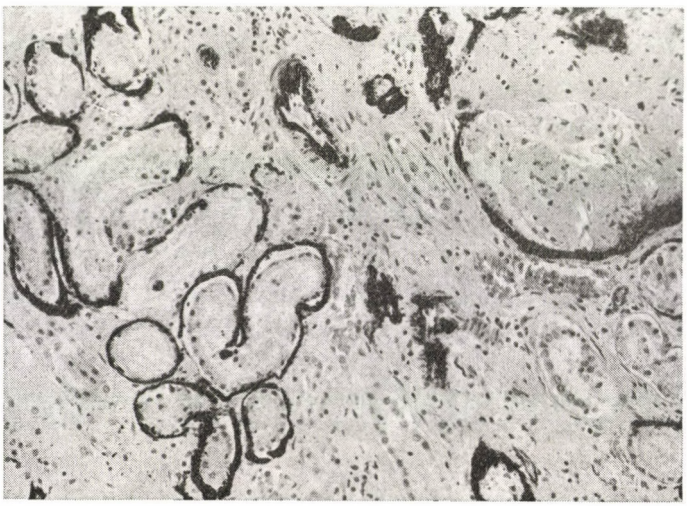

Fig. 9. Deposition of calcium in Bowman's capsule and along the basement membrane of tubules. Amyloidic contracted kidney. Kossa's staining, approx. ×206

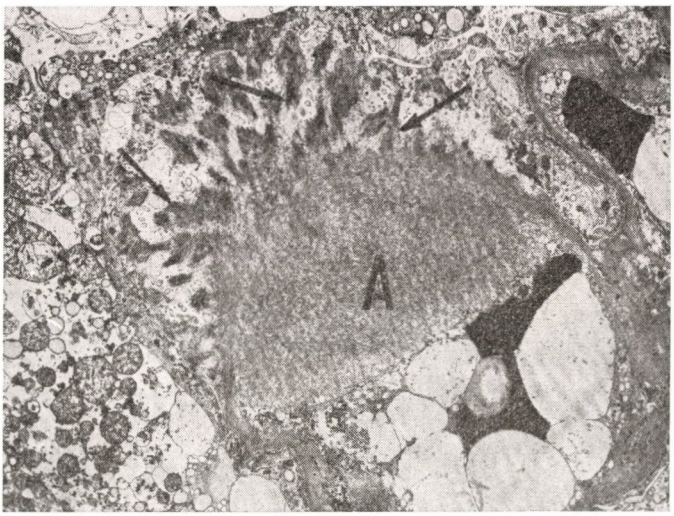

Fig. 10. Amyloid deposit of subendothelial localization (A) and radial bundles of amyloid (arrow) between the foot processes of podocytes. Between them, note the loosened basement membrane (BM) of the capillary. Transmission electron micrograph of necropsy material, ×3600

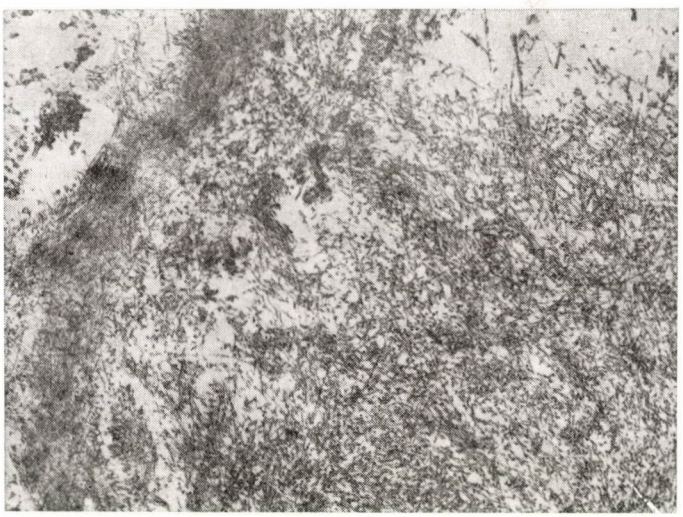

Fig. 11. The light-microscopically homogeneous amyloid deposit is of filamentous structure. Transmission electron micrograph of necropsy material, ×18,000

of the tubule. On the other hand, other tubules showed hyperplasia (enlargement, and dilatation of the lumen). The intertubular connective tissue widened, particularly between atrophic or destroyed nephrons. In the fibrous or fibrillary connective tissue, an infiltration with mononuclear cells (mainly plasma cells) was seen; the extent of the infiltration was variable in each area. In the

proliferated connective tissue of the renal interstitium, primarily between the collecting tubules in the medulla, the deposition of a substance characterized by greenish polarization light typical of amyloid was seen in smaller or larger areas. In Bowman's capsules of numerous glomeruli and in basement membranes of many tubules, the deposition of granules consisting of calcium compounds was seen, indicating prolonged uraemia (Fig. 9).

In cases Nos 1, 2, 3 and 4 amyloid deposition was not seen in other organs (liver, spleen, pancreas etc.). In case No. 5, amyloid was observed in the wall of the small arteries of the spleen, in addition to renal glomeruli and

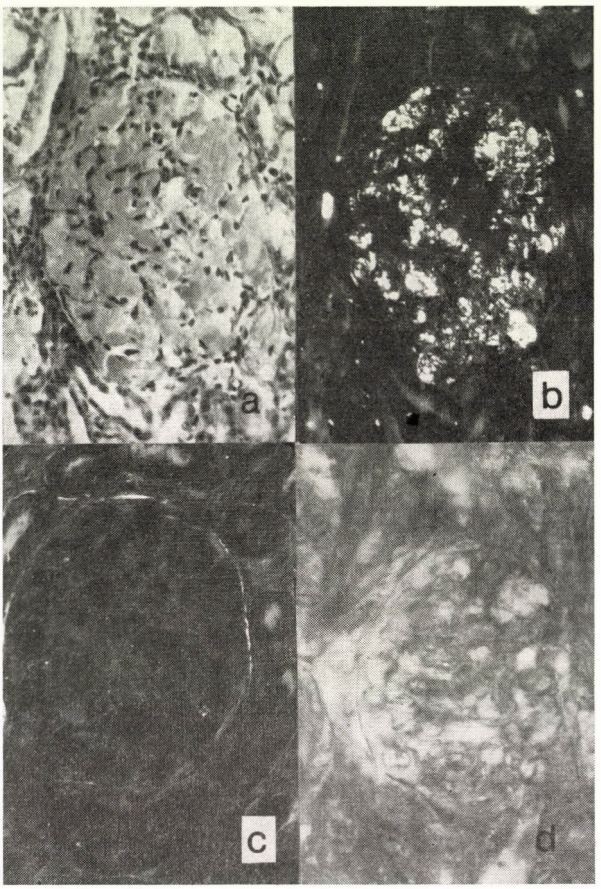

Fig. 12. Studies on the nature of amyloid deposited in the glomerulus. Congo red staining, sections covered with gum arabic, approx. ×220. *a* Light-microscopic photograph. Amyloid is seen in the form of homogeneously staining deposits. *b* Polarization-microscopic photograph of the same glomerulus. Amyloid shows strong birefringence. *c* Polarization-microscopic photograph taken after performic acid pretreatment. Amyloid stains with Congo red but it has lost its birefringence. *d* Light-microscopic photograph. Trypsin treatment performed after oxidation with a mixture of potassium permanganate and sulphuric acid solutions resulted in complete digestion of amyloid

smaller intertubular areas of amyloid deposition found in the renal interstitium.

Transmission electron microscopy revealed that the material deposited in the glomeruli had a filamentous and fibrillar structure typical of amyloid (Figs 10 and 11). In other areas of the kidneys (in blood vessels other than glomerular capillaries, and along the basement membrane of tubules) amyloid deposition was not observed in any of the cases.

In studies on the nature of amyloid, after performic acid pretreatment of deparaffinized sections, in preparations stained with Congo red and covered with gum arabic, the material deposited in the glomeruli lost its birefringence in all five cases, typically of the structurally labile secondary amyloid (Figs 12*a*, *b*, *c*). Similarly, the amyloid focally distributed in the renal interstitium and deposited in the wall of arterioles of the spleen did not show birefringence in sections prepared (as described above) from the organs of dog No. 5.

After pretreatment with a mixture of diluted potassium permanganate and sulphuric acid solution, and digestion with trypsin for 2 and 24 h, the amyloid which had deposited in the glomeruli and, in Case No. 5, in other areas of the kidney, was digested after 2 h digestion in all cases, as typical of secondary amyloid (Fig. 12*d*).

Discussion

Amyloid is considered to be a substance of protein nature (glycoprotein) which deposits extracellularly and shows marked variation between species, individuals and cases. Several authors have suggested the importance of immunobiological processes in the production of amyloid and in the pathogenesis of amyloidosis. Amyloid has been considered to result from antigen-antibody coupling, and its appearance in different organs has been postulated to indicate the accumulation of antigen-antibody complex (Smith and Jones, 1961; Vazquez and Dixon, 1956). This postulation was supported by observations that amyloidosis occurred after persistent or recurring antigenic stimuli in both spontaneous and experimental cases (Rothbard and Watson, 1954; Theilum, 1954). It was also demonstrated that globulin concentrations of the serum increased in both cases (Giles and Calkins, 1958; Huestis and Jaeger, 1960). By immunofluorescent procedures it was confirmed that amyloid deposits contained large amounts of globulin (Vazquez and Dixon, 1956).

Depending upon its characteristics (molecular weight etc.) and rate of production, the produced antigen-antibody complex deposits in different sites, to different degrees and intracellularly (in the wall of smaller blood vessels and in the connective tissue surrounding them, and along the basement membranes in different organs).

Presumably, mechanisms other than those mentioned above also play

significant roles in the production of amyloid. The variability of these mechanisms, which are yet insufficiently known, is expressed also in the complex composition of amyloid.

In amyloidosis, the kidneys (and particularly their glomeruli) are the affected organs. The conditions predominating in these sites (anatomical structure of glomeruli, the relatively high blood pressure ensuring ultrafiltration) predispose glomeruli for deposition of different substances including amyloid.

As it was observed also in our cases, amyloid deposits primarily in the glomeruli and glomerular capillaries. In the dog, extraglomerular amyloid deposition (in the wall of small blood vessels outside the glomeruli, sometimes in the interstitium and exceptionally along the basement membranes of tubules) can be observed only in more severe cases of amyloidosis.

In both man and animals, amyloid initially forms deposits in the glomeruli between the basement membrane of capillaries and the endothelial cells (subendothelially), as a characteristic, light-microscopically homogeneous and electron-microscopically filamentous substance (Osborne et al., 1968, 1969). Primarily this was found also in our cases. However, in some cases deposition of amyloid in the basement membrane of capillaries was also observed. This led to structural disintegration of the basement membrane. In some sites, amyloid appeared also in the form of radial bundles between the projections of podocytes (subepithelially).

In preparations stained with Congo red, covered with gum arabic and examined in polarization microscope, amyloid shows intensive birefringence (additive topo-optical reaction), while collagen is isotropic or exhibits mild negative birefringence (inverse topo-optical reaction). This method is particularly suitable for the selective optical detection of the finest amyloid structures (Romhányi, 1971, 1979).

On the basis of ultrastructural stability studies, two types of amyloid can be distinguished: *(i)* the structurally labile amyloid, e.g. the (generalized) secondary amyloid; and *(ii)* the structurally stable amyloid, e.g. in case of (generalized) primary amyloidosis, local and senile amyloidosis. This classification is consistent with that of Benditt and Eriksen (1971), in which A and B types of amyloid were discerned.

Two major directions of stability studies are known: studies on the resistance *(i)* to performic acid, and (*ii*) to proteolytic enzymes. Following treatment with performic acid, the structurally labile amyloid loses its birefringence, due to the break-up of disulphide bonds. On the other hand, structurally stable amyloid retains its birefringence even after performic acid treatment (Romhányi, 1979).

Resistance to proteolytic enzymes including trypsin is a characteristic feature of amyloid fixed in formaldehyde solution. If the section is subjected

to pre-oxidation of medium degree (e.g., in the present cases to treatment with a mixture of diluted potassium permanganate and sulphuric acid solutions), secondary amyloid of all organs will be digestible with trypsin, while primary amyloid will retain its resistance to trypsin digestion (Romhányi, 1972).

Relying upon the results of tests on the resistance of amyloid to digestion with performic acid and trypsin, it may be stated that the cases included in the present studies represented secondary amyloidosis.

Although at necropsy, apart from renal amyloidosis, no lesions indicative of disease were found in our cases, the stability tests unanimously supported the diagnosis of secondary amyloidosis. This suggests that a disease, presumably of inflammatory nature, must have taken place earlier, so that its lesions were already unobservable at the time of necropsy.

Occasionally the extensive subendothelial deposition of amyloid in the glomeruli may even cause obstruction of capillary lumina and discontinuation of ultrafiltration, due to the fact that glomerular capillaries become impenetrable for the blood. If this condition becomes extensive, it may lead to acute kidney failure (renal insufficiency).

However, the condition characterized by damage of the filtration barrier, which has a decisive role in ultrafiltration of blood plasma, seems to be far more frequent. The damage is due to subendothelial deposition of amyloid. As a consequence, the filtration barrier becomes permeable for high-molecular-weight proteins, resulting in the appearance of protein (primarily albumin) in the urine. Although tubular epithelial cells may reabsorb considerable amounts of protein, similar amounts (30–34 g/l) of protein (of which 6.7–14.2 g/l is albumin) are excreted from the organism in the secondary urine (Watson, 1971). Due to the considerable (up to even 10 g daily) total protein loss (as much as half of which may be albumin), the nutritional status of the animals may undergo marked deterioration (Osborne et al., 1969). The prolonged albuminuria leads to hypalbuminaemia. Deficiency in albumins, the plasma constituents most important in maintaining colloidal-osmotic pressure, results in oedema in several parts of the body (Lapras, 1979; Osborne et al., 1968, 1969).

The protein present in the primary urine is partly reabsorbed by the tubular epithelial cells (primarily those of the proximal tubules), which will be seen as hyaline degeneration of the cells. Reabsorption may reach such an extent that the tubular epithelial cells overloaded with lysosomal protein drops become incapable of any other function. The disturbance of the selective reabsorption process may further complicate the process of secondary urine formation and the disorders of blood plasma homeostasis.

The hypalbuminaemia caused by disturbed renal function results in the formation of oedema in the subcutaneous tissues and in the body cavities

(so-called renal oedema). Certain authors (Opitz, 1966) noted that, in case of hypalbuminaemia, oedema developed far less frequently in dogs than in human beings, as compared to its expected occurrence. In dogs, formation of diffuse oedema may be reckoned with if the albumin concentration of the plasma drops below 8 g/l (Yamauchi et al., 1964).

In addition to hypalbuminaemia, hypercholesteraemia is also characteristic of the condition. The cause of the development of hypercholesteraemia is yet insufficiently known (Osborne et al., 1968, 1969; Slauson and Gribble, 1971).

We observed massive proteinuria in all five cases. Two dogs (Nos 1 and 2) showed considerable deterioration of nutritional status and diffuse oedema, both attributable to the prolonged protein loss. Although the necropsy material did not allow the examination of all parameters involved, oedema was considered to be a symptom of the nephrotic syndrome.

In two dogs (Nos 3 and 4), in addition to amyloidosis of renal glomeruli and the resulting nephrotic syndrome, thrombosis occurred: in both branches of the pulmonary artery in one of the dogs, and in the pulmonary veins and in the vena cava caudalis in the other.

In this context, we refer to the studies of Slauson and Gribble (1971), who found 52 cases of renal amyloidosis among 61 canine amyloidosis cases. Of the 52 dogs, 20 were found to have thrombosis in different organs; occasionally blood vessels of several organs (pulmonary artery, coronaries of the heart, arteries of the spleen and kidneys, arteria mesenterica, arteria iliaca, a. brachialis and vena portae) were found to contain thrombi simultaneously. According to Slauson and Gribble (1971), thrombus formation accompanying renal amyloidosis is a consequence of the nephrotic syndrome.

Apart from glomerular amyloidosis, nephrotic syndrome may associate with other glomerulopathies. Therefore, the risk of thrombosis is to be reckoned with also in these cases, as it was pointed out by Müller-Peddinghaus et al. (1978) in the case of the dog. Thrombus formation associated with nephrotic syndrome is known to occur not only in animals but also in human beings.

Also in our opinion, thrombus formation observed in the present cases in the pulmonary artery, pulmonary veins and vena cava caudalis is a consequence of renal amyloidosis and the resulting nephrotic syndrome.

The pathomechanism of thrombosis observed in cases of glomerular amyloidosis is not completely understood yet; for its clarification, changes of all factors involved in the mechanism of blood coagulation should be studied. However, certain phenomena are worth mentioning: among others, the fact that the colloidal-osmotic pressure of the blood plasma undergoes a significant decrease due to hypalbuminaemia, losses occur in the quantity of plasma fluids, resulting in thickening of the blood. This leads to increased viscosity and reduced flow rate of the blood. Because of the latter, the cellular elements

of the blood (including thrombocytes) get to the periphery of the circulation. The adhesion between the endothelial cells of blood vessels and the cellular elements of blood offers favourable conditions for the initial processes of thrombus formation (Slauson and Gribble, 1971). In cases of glomerular amyloidosis, hyperfibrinogenaemia, another phenomenon favouring thrombotic tendency, is also frequently observed (Slauson et al., 1970; Slauson and Gribble, 1971).

In addition to the above facts, development of thrombotic tendency may be furthered by the production of larger amounts of alpha-globulin in the organism, as a reaction to albumin loss and decrease of colloidal-osmotic pressure. This protein was shown to possess antifibrinolytic properties (Jacobsson, 1955). Based mainly upon experiences gained by human medicine, the increased lipid (primarily cholesterol) content of blood plasma, which is a concomitant feature of the nephrotic syndrome associated with amyloidosis, is considered to be a significant factor predisposing to thrombosis (Mustard et al., 1964). In the process of thrombus formation associated with the nephrotic syndrome, the injuries of the intima of blood vessels, apart from the accompanying hypoxaemic phenomena, are not considered characteristic.

It is obvious from the phenomena indicated above that in the development of thrombosis the most important part is played by the increased coagulability (hypercoagulability) of blood which may be supported by the dysfunction of the fibrinolytic system (Slauson and Gribble, 1971). In all probability, other factors may also have a role in inducing thrombotic tendency in nephrotic syndrome, apart from the involvement of the factors described above, the degree of which varies from case to case.

Glomerular amyloidosis is usually accompanied by regressive changes of tubular epithelial cells and atrophy and destruction of nephrons. Together with the changes of tubules, infiltration of the renal interstitium with inflammatory cells can be observed in the initial stages, as a reactive process. Subsequently, during the process of reparation, destroyed nephrons are replaced by collagenic connective tissue (amyloid nephrocirrhosis, contracted kidney) which also leads to renal insufficiency (prolonged uraemia) (Dahme and Weiss, 1978), as it was observed in one (No. 5) of the present cases.

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PURIFICATION AND PARTIAL CHARACTERIZATION OF A PROTEASE INHIBITOR OF *PROTEUS VULGARIS*

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The trypsin- and chymotrypsin-inhibiting factor of cell extracts of *Proteus vulgaris* was studied. The inhibitor was purified by affinity chromatography. It was confirmed by enzymatic pretreatment that identical (or very closely adjacent) sites were responsible for the inhibition of both trypsin and chymotrypsin. The molecular weight of the inhibitor was in the range of 30,000–32,000 as determined by gel chromatography.

As opposed to other protease inhibitors, bacterial protease inhibitors have been little studied. Høyem and Skulberg (1962) detected a trypsin inhibitor in supernatants of *Clostridium botulinum* cultures, while Brecher and Pugatch (1969) demonstrated the presence of a non-dialysable, heat-stable trypsin- and chymotrypsin-inhibiting factor in extracts of sonicated *E. coli* cells.

Fossum's work (1970) was a major contribution to the studies on bacterial protease inhibitors. It included extensive studies on Gram-positive and Gram-negative bacteria. Fossum (1970) found that while sonicated cells of Gram-positive bacteria did not show inhibitory activity, among Gram-negative bacteria the extracts of sonicated cells of *Proteus mirabilis*, *Proteus vulgaris*, *E. coli*, a *Klebsiella* sp., *Serratia marcescens* and *Pseudomonas aeruginosa* inhibited the activity of trypsin, chymotrypsin A and of extracellular proteases of certain microorganisms. Electrophoretically, the trypsin- and chymotrypsin-inhibiting factor appeared as a single band.

The present work was aimed at studying the trypsin- and chymotrypsin-inhibiting factor of *Proteus vulgaris*, a Gram-negative bacterium species with relatively high inhibitory activity.

Materials and methods

Preparation of inhibitor-containing crude extract

A modification of Fossum's method (1970) was used. The strain was inoculated on nutrient agar plates in Petri dishes, incubated at 37 °C for 2 days and the colonies were removed from the surface of the agar by washing.

The cells were washed 3 times in physiological saline, centrifuged at 19,000 *g* for 10 min and suspended in 50 times their wet weight of distilled water. Subsequently, the cells were sonicated in an MSE sonifier (1.2 A) for 40 min. The inhibitor was heat-stable, thus its further release from the cells could be facilitated by boiling for 5 min. After centrifugation at 19,000 *g* for 40 min, the supernatant was examined for inhibitory activity (it had been freeze-stored until used). The procedures described above were performed in two parallel series.

Determination of enzyme and inhibitory activities

Enzyme activity was measured spectrophotometrically, in TRIS-buffer of pH 7.8. The trypsin- and chymotrypsin-activities were determined by the method of Schwert and Takenaka (1955) and by that of Hummel (1959), respectively. Since in preliminary experiments the time of formation of the enzyme–inhibitor complex had been found to be 1–1.5 min, a preincubation time of 2 min was employed in all determinations. Inhibitory activities were expressed as the rate of enzyme activity decrease.

One unit (U) of enzyme activity is defined as the amount of enzyme hydrolysing 1 μmol substrate per min. One unit of inhibitory activity is defined as the amount of inhibitor which inactivates one unit of enzyme.

If chymotrypsin pretreatment was used, the inhibitor was reacted with chymotrypsin A having 50 times as high activity as that of the inhibitor. Ten minutes later, the inhibitor–chymotrypsin complex thus formed was examined for trypsin-inhibiting activity. Trypsin pretreatment was performed in a similar manner: the inhibitor was reacted with trypsin treated with tosyl-phenylalanine chloromethyl ketone (TPCK trypsin, which is completely free of chymotrypsin activity). The trypsin-inhibiting activity of the complex was determined after an incubation of 10 min.

Affinity chromatography

Chymotrypsin A (bovine pancreatic, 4×crystallized; British Drug House, BDH) was coupled to CNBr-activated Sepharose 4B (Pharmacia) according to the method of Feinstein (1971). The conjugate was packed into a column of 0.9×10 cm and was equilibrated with 0.1 mol borate buffer (pH 9.0). The extract was eluted with borate buffer and the bound inhibitor was desorbed with 0.05 N HCl (pH 1.6). Fractions of 2 ml were collected.

Gel chromatography

Sephadex G-75 gel (Pharmacia) was used in a column of 2.6×40 cm, with 0.1 mol/l TRIS-HCl buffer (pH 8.0) containing 0.2 mol NaCl/l used for equilibration and elution, collecting 5-ml fractions. The column was calibrated

with proteins of known molecular weight: bovine serum albumin (Calbiochem M_r 67,000), soybean trypsin inhibitor (BDH, M_r 21,500), cytochrome *c* (Koch-Light, M_r 12,400), *Cysticercus pisiformis* protease inhibitor (own preparation, Németh and Juhász, 1980; M_r 7,100). The molecular weight was calculated from the following regression equation:

$$\log M_r = 5.8084 - 1.5902\, K_{av} \quad (r^2 = 0.9989)$$

All determinations were carried out in triplicate.

Results

The crude extracts possessed neither trypsin nor chymotrypsin activity. The inhibitory activities are shown in Table I.

For purification, 4 ml volumes of both extract 1 and 2 were applied on a Sepharose-chymotrypsin column. The results are indicated in Table II.

No inhibitory activity was demonstrable in the eluate obtained by elution with borate buffer. Upon elution with HCl, both trypsin and chymo-

Table I

Inhibitory activities of crude extract and bacterial cell mass of *Proteus vulgaris*

	Bacterial cell mass g	Volume ml	Trypsin inhibitor		Chymotrypsin inhibitor	
			U/ml	U/g of bacterial cell mass	U/ml	U/g of bacterial cell mass
Extract 1	0.225	11.25	130	6,500	176	8,800
Extract 2	0.190	9.50	142	7,100	208	10,400

Table II

Recovery of the trypsin and chymotrypsin inhibitor from extracts of *Proteus vulgaris* by affinity chromatography

	Inhibitory activity (U) for			
	trypsin		chymotrypsin	
	extract 1	extract 2	extract 1	extract 2
Crude extract (4 ml)	520	568	704	1132
Fractions eluted by borate buffer (40 ml)	0	0	0	0
Fractions eluted by HCl (4 ml)	340	344	485	715
Recovery (%)	65.4	60.6	68.9	63.2

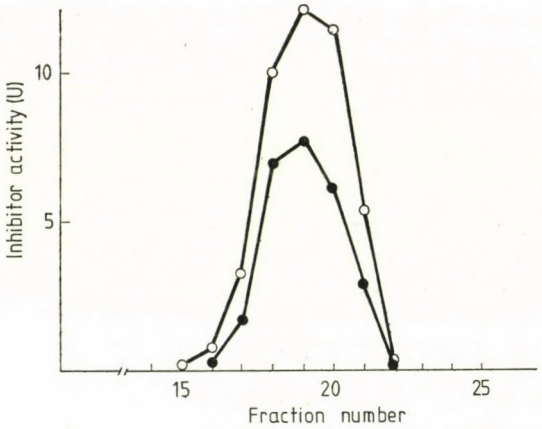

Fig. 1. Chromatography of the protease inhibitor of *Proteus vulgaris* on Sephadex G-75 column. The trypsin- and chymotrypsin-inhibiting activities of the 2-ml sample applied to the column were 170 and 242 U, respectively. ●—●, trypsin inhibitory activity; ○—○, chymotrypsin inhibitory activity. Absorbance at 280 nm was not measurable

trypsin inhibitory activities were desorbed from the gel, with an efficacy of 60–70%. The high degree of purification achieved by affinity chromatography was indicated by the immeasurably low E_{280} value of the HCl eluate, as opposed to values obtained for the first and second extract (0.254 and 0.310, respectively).

Our further studies were performed to elucidate the closer structure of the inhibitor; namely, to determine whether the trypsin- and chymotrypsin-inhibiting activity can be attributed to the same active site or whether there are two separate active centres localized at different sites of the molecule.

After chymotrypsin pretreatment, the inhibitor solution purified by affinity chromatography showed no trypsin-inhibiting activity. Similarly, pretreatment of the inhibitor with TPCK trypsin resulted in complete disappearance of chymotrypsin-inhibiting activity.

The inhibitor was studied by gel chromatography, using a Sephadex G-75 column (Fig. 1), and it eluted as a single symmetrical peak ($K_{av} = 0.83$), which fact indicated its homogeneity. The molecular weight of the inhibitor was 30,000–32,000, as estimated by comparing it to standard substances of known molecular weight.

Discussion

The subject of our studies was the trypsin- and chymotrypsin-inhibiting factor which was first described by Fossum (1970) as an electrophoretically homogeneous substance. The present results confirmed the homogeneous

nature of the inhibitor from the functional point of view, since during purification by affinity chromatography the inhibition of both homologous enzymes was found to be the property of the same molecule. In addition to the trypsin and chymotrypsin inhibitor, Fossum (1970) separated electrophoretically another factor inhibiting bacterial proteases. This latter inhibitor was not the subject of the present studies.

By affinity chromatography, the inhibitor was obtained in a highly purified form.

By enzymatic pretreatment, evidence was produced that the inhibition of the two homologous enzymes is the property of the same active site, or of two very closely adjacent sites, of the inhibitor molecule; therefore, the inhibitor is not of the "double-headed" type.

Among natural protease inhibitors, the ones inhibiting both trypsin and chymotrypsin and similar to the inhibitor of *Proteus vulgaris* are of frequent occurrence: e.g. the protease inhibitors of plants, the basic pancreatic trypsin inhibitor, the inhibitors of egg white etc. (Kassel, 1970) and the inhibitors of parasitic helminths (Juhász, 1982).

The molecular weight of the inhibitor was determined by gel chromatography. Because of the estimated molecular weight of 30,000–32,000, therapeutic application of the inhibitor may meet with difficulties, for it may behave as an antigen, causing anaphylactic side effects.

Studies on the biological function of the inhibitor are reported in a separate paper (Juhász and Prohászka, 1983).

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CHANGES IN THE PLASMA CONCENTRATIONS OF THYROID HORMONES AND SEXUAL STEROIDS DURING FORCED MOLT OF MALE AND FEMALE DOMESTIC CHICKENS

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Changes in the thyroid and gonadal function of male and female domestic chickens were followed up during the period of forced molt induced by 2-day deprivation of feed, drinking water and light, plus cold exposure. The layers showed a significant increase in plasma progesterone during the period of feather loss, followed by a gradual decrease, whereas plasma 17-β-oestradiol persisted at a low level throughout. The plasma thyroxine rose initially along with progesterone, persisted at an elevated level during the molt period, and tended to decrease as the new feathering was acquired. The triiodothyronine concentration tended to decrease initially, then tended to increase during feather growth.

The roosters showed an initial drop of plasma testosterone, which remained at a low level subsequently. The plasma thyroxine concentration tended to increase parallel to testosterone decrease. The plasma triiodothyronine concentration changed similarly to the layers, but in a not significant degree.

These experimental observations suggest the responsibility of altered thyroid–gonadal interaction for molt control in the male and female domestic chicken. In layers, increased concentrations of progesterone and thyroxine induced feather loss, through their effect exerted on the papillae. In roosters, the increase of thyroxine concentration and its effect on the feather papillae were rendered possible by the low testosterone level.

At the time of new feather outgrowth, both male and female chickens showed high plasma levels of thyroxine and triiodothyronine, and low plasma levels of sexual steroids.

Molting, a characteristic physiological process in birds, has been studied extensively by various experimental approaches in both domestic and wild birds. Investigations into its endocrine control had initially suggested the primary responsibility of the thyroid gland (Brake et al., 1979; Cole and Hutt, 1928; Davis and Davis, 1954; Glazener and Jull, 1946), but later evidence of the major involvement of other endocrine organs, such as the pituitary gland (Juhn and Harris, 1956), adrenals (Brake et al., 1979; Perek and Sulman, 1959), or gonads (Harris and Shaffner, 1957; Juhn and Harris, 1956, 1958; Tanabe et al., 1956) led to postulate an endocrine cooperation. Investigations into the influence of thyroid–adrenal interaction (Brake et al., 1979) failed to detect an unequivocal interrelationship. With the experimental facts in mind, a functional interaction between the thyroid gland and gonads seems to represent the underlying mechanism of molting, but the substantiation of this hypothesis requires investigations by hormone-analytical methods (competitive protein-binding analysis, radioimmune analysis) which, as far as we are informed, have not yet been performed to this end.

To obtain more information on the problem, we studied fluctuations in the plasma thyroid and gonadal hormone levels of male and female domestic chickens in the course of forced molt.

Materials and methods

Two parent flocks of the Eurohyb breed, aged 57 and 67 weeks, respectively, were used in two experimental series. Blood samples were collected from a representative group of 9 birds on each occasion, at weekly intervals from the beginning of molting till the new feathering was acquired. In the first experimental series 6 roosters were sampled along with the layers.

At the beginning of forced molt, egg production was low (21 and 14%) in both flocks, and the sexual activity of the roosters was considerably reduced. The daily ration of the female birds consisted of 0.162 and 0.171 kg layer feed, respectively, plus 0.014 kg barley grains; this level of feeding increased over that consistent with the applied management technique. A constant photoperiod of 18 h daily illumination plus 6 dark hours was employed. The ambient temperature of the layer houses was maintained at 12–16 °C during the last stage of the production period.

Molt was induced by depriving the birds of feed, drinking water, daily illumination and heating for 2 days. The level of feeding was risen to the daily ration of 0.140 kg layer feed + 0.010 kg barley, as scheduled under the given management technique, only by 6 weeks of experiment. Table I shows the feed and drinking water supplies, and light regimens applied during the molting period.

Blood samples were withdrawn from the brachial vein once weekly at the different stages of forced molt. The first blood sampling was carried out before the beginning of the experiment. The second and third blood samples were withdrawn after the loss of contour feathers and down feathers. Next week the down feathers grew out again in the cervical and thoracic region but the contour feathers were still lacking. At the fifth sampling, the down feathers were present everywhere except in the tail region, and of the contour feathers the pinions had reappeared. By the last blood sampling all birds had acquired a complete new feathering. Forced molt had a less drastic effect on roosters than on layers. The male birds did not exchange all primary feathers, and the loss and reappearance of the secondary feathers was less sharply delineated in time. The forced molt of the roosters resembled naturally occurring molt in many respects.

Forced molt resulted in termination of egg production within 1–2 days. The egg yield reached 50% of the original level during the 11th week after the beginning of molting.

Table I

Schedule of feeding, watering and lighting during the first six weeks of forced molt

Days of forced molt	Amount of food day/animal	Access to water h	Lighting h
1–2	—	—	—
3–9	0.05 kg barley	2	2
10–15	0.07 kg barley	4	4
15–18	0.07 kg barley	6	6
19–21	0.04 kg barley	8	8
	0.04 kg barley		
22–28	0.04 kg barley	10	10
	0.04 kg starter feed		
29–32	0.04 kg barley	12	12
	0.10 kg laying feed		
33–35	0.04 kg barley	12	12
	0.12 kg laying feed		
36–39	0.04 kg barley	12	12
	0.13 kg laying feed		
40–42	0.04 kg barley	12	12
	0.14 kg laying feed		

The plasma levels of thyroid hormones (triiodothyronine, thyroxine) were determined as proposed by Pethes et al. (1977, 1978). Of the ovarian hormones progesterone was determined according to Abraham et al. (1971), and 17-β-oestradiol according to Mikhail et al. (1972). Testosterone assay was carried out by the method of Jallageas and Assenmacher (1972).

Results

A. *Layers*

Group I. Plasma progesterone had increased significantly over the control by the 5th day of forced molt, then tended to decrease; the concentration decrease found at the end of the experiment also was significant. The plasma level of 17-β-oestradiol remained evenly low throughout the period of forced molt (Fig. 1).

The plasma thyroxine level tended to rise initially, persisted at an evenly high level until the birds acquired feathering again, then tended to decrease significantly ($P < 0.001$). Plasma triiodothyronine decreased significantly in the early stage of forced molt, increased significantly when feather outgrowth

began, and tended to decrease again, to a significant degree ($P < 0.05$), by the end of the experiment (Fig. 2).

Group II. Changes in progesterone level follow exactly the pattern obtained in the case of the former experimental group. The suddenly rising first part reaches significant difference, later on the level of progesterone drops and turns out to be significantly lower. The concentration of 17-β-oestradiol exhibits a low and non-fluctuating pattern also in this group (Fig. 3).

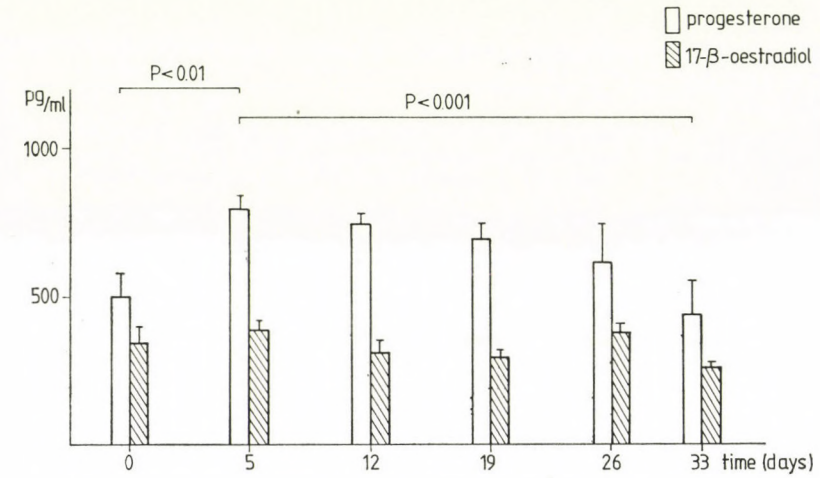

Fig. 1. Changes of progesterone and oestradiol levels of hens (Exp. 1; n = 9)

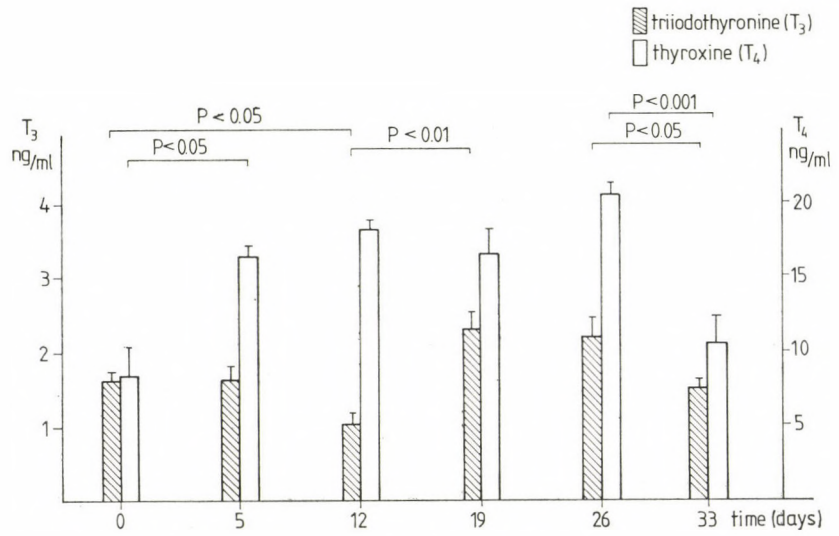

Fig. 2. Changes of thyroxine and triiodothyronine levels of hens (Exp. 1; n = 9)

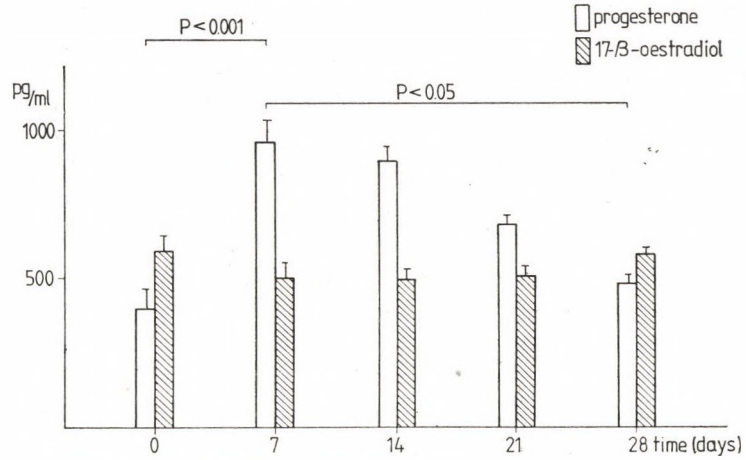

Fig. 3. Changes of progesterone and oestradiol levels of hens (Exp. 2; n = 9)

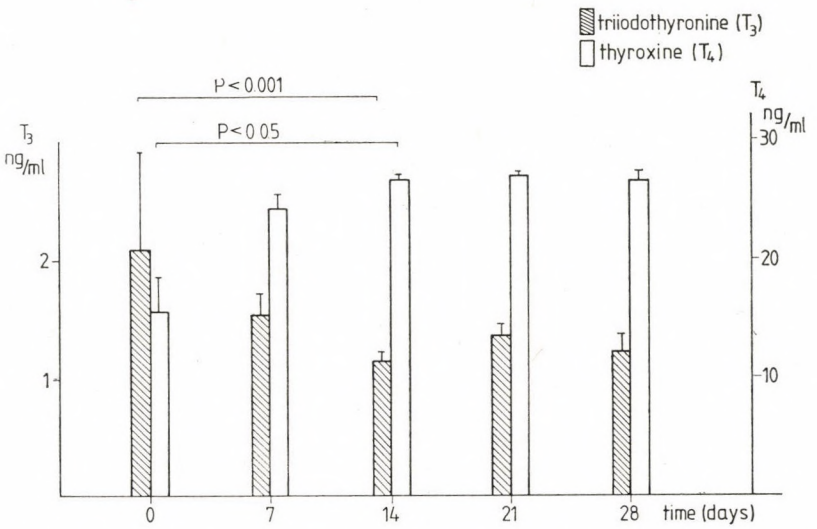

Fig. 4. Changes of thyroxine and triiodothyronine levels of hens (Exp. 2; n = 9)

Plasma thyroxine tended to rise from the beginning of the experiment; owing to great individual variation, increase over the control was not significant until the third week, but then a plateau followed, as in the first experiment. The initial significant decrease in plasma triiodothyronine was also observed in the second experiment, but the concentration increase ensuing at the beginning of feather growth was not significant in that instance. Since the last blood sampling could not be carried out in the second experiment for technical reasons, no evidence was obtained of the supposed final concentration decrease of the two thyroid hormones (Fig. 4).

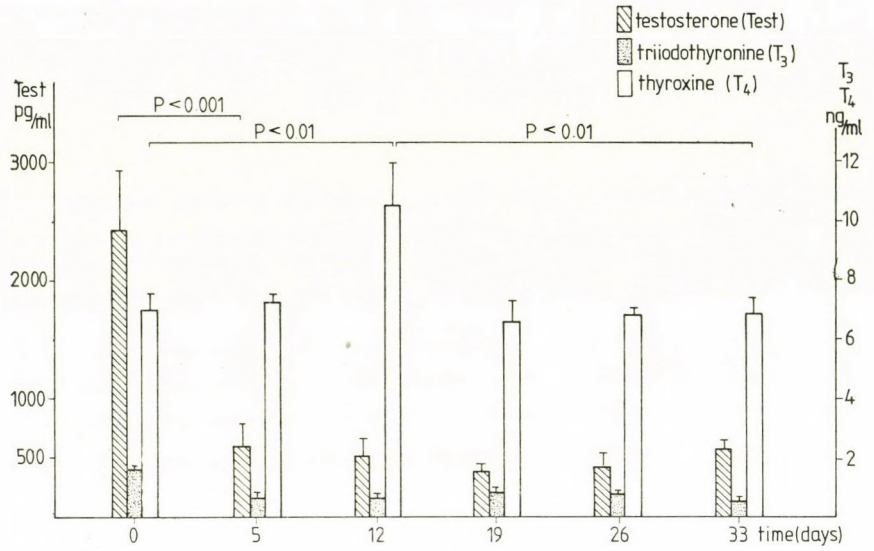

Fig. 5. Changes of testosterone, thyroxine and triiodothyronine levels of roosters (n = 6)

B. *Roosters*

Group II. The plasma testosterone showed an abrupt significant decrease at the beginning of forced molt, and remained at a reduced level throughout.

The initial significant rise of plasma thyroxine was followed by a gradual decrease, which reached a significant degree by the conclusion of feather exchange. Triiodothyronine tended to decrease when molting began, and rose slightly when feather outgrowth became more intensive (Fig. 5).

Discussion

The plasma progesterone concentration of layer hens is generally low, except for a peak observed 4–7 h before ovulation (Furr et al., 1973; Laque et al., 1975; Péczely et al., 1980; Shababi et al., 1975). The basic level is represented by the control groups. The concentration increases observed during forced molt made up only one third or one fourth of the pre-ovulation peak. This can probably account for lack of ovulation, and consequent absence of oviposition during forced molt. The relatively high progesterone level tended to decrease later. The important role of progesterone in molting is indicated by the fact that it was found to induce molt in chicks and hens in experimental conditions (Adams, 1956; Harris and Shaffner, 1957; Juhn and Harris, 1956). Although the last-cited authors used pharmacological doses of pro-

gesterone, it may be inferred in view of the pertinent literary data that increase in progesterone concentration is probably one of the factors responsible for the induction of molting.

Further to low plasma progesterone, the evenly low 17-β-oestradiol concentration also was indicative of a reduced ovarian activity. Certain authors (Petterson and Common, 1972) observed regular, while others (Laque et al., 1975; Péczely et al., 1980) irregular peaks, whose precise role is still obscure. Little information is also available on the role of sexual steroids in molting (Farner and King, 1972). A molt inhibitor effect of exogenous oestrogen has been reported. Our own experimental observations have indicated that 17-β-oestradiol is not involved in the control of certain molting stages, since its plasma concentration did not change during the forced molt process.

However, a permissive effect of low oestradiol on the molt-controlling action of progesterone cannot be ruled out with certainty.

Thyroid involvement in molt control has long been postulated, but some evidence against it has also been reported (Sulman and Perek, 1947; Wilson and Farner, 1960).

Decrease in the plasma testosterone concentration of roosters indicated a reduced testicular activity. This accords well with the observation that castration does not prevent molting (Morton et al., 1962), but exogenous testosterone (Kobayashi, 1954; Vaugien, 1955) or testosterone implantation does (Farner et al., 1980). Wild male birds also had low plasma testosterone levels during the molt period (Temple, 1974).

Brake et al. (1979) observed in birds fasted during the initial period of forced molt a decrease in plasma thyroxine with the onset of feather loss, a subsequent increase, and decrease again after fasting was terminated. Against this Assenmacher (1958) was able to produce serial molts by thyroxine treatment, and May (1977) observed an abrupt rise of plasma thyroxine in fasting birds. In our own experiments, the considerable increase in plasma thyroxine concentration may have been due to the joint effect of fasting and cold environment, and may have been, in our view, directly or indirectly, responsible for the induction of feather loss. Decrease in plasma triiodothyronine, as found by us, has also been observed by other investigators. May (1977) and Brake et al. (1979) unequivocally found a decrease in plasma triiodothyronine during the fasting period. Starvation may have been of a lesser degree in roosters than in layers, since the former take up more feed than the layers, consistent with their stronger body conformation, as suggested also by the lesser decrease in T_3.

The layers showed a distinct, whereas the roosters a less distinct, increase in plasma triiodothyronine during the period of feather outgrowth. These facts support Höcker's (1967) implication that an increase in T_3 secretion enhances feather growth.

By contrast, Brake et al. (1979) arrived at the conclusion that the T_3 concentration increases, rather than decreases, in the initial stage of forced molt, and the increased concentration is responsible for feather loss. Our own experimental results suggest that molt-associated alterations in the gonadal–thyroid functional balance characteristic of the adult male and female chicken are of decisive importance. This functional interrelationship had been inferred earlier by Himeno and Tanabe (1957).

Significant increase in plasma progesterone along with thyroxine during the early stage of molting seems to develop a direct synergistic action on the feather papillae and to account thereby for feather loss in female birds. This assumption agrees well with the observation of Harris and Shaffner (1957) that simultaneous feeding of thyreoprotein enhanced the molt-inducing effect of exogenous progesterone.

Decreased testicular function, observed in roosters during the early stage of molting, was accompanied by an increase in thyroid function. Association of decrease in plasma testosterone with an increase in thyroid hormone levels also occurs in male birds (Jallageas and Assenmacher, 1972, 1974; Péczely et al., 1979).

We believe that while triiodothyronine enhances feather growth, plasma progesterone, 17-β-oestradiol and testosterone remain at low levels.

The available experimental observations do not permit unequivocal conclusions on the mechanism of molting when analysed by aspects of comparative physiology, owing in all probability to species-specific differences in the hormonal control of the molting cycle (Payne, 1972). Further investigations into the problem should be based, as proposed by Farner (1981), on studies of the hormone receptors of feather follicles by radio receptor assay.

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WATER AND ELECTROLYTE METABOLISM IN SHEEP*

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In three series of experiments, the effect of NaCl and water loading was investigated on the electrolyte and water metabolism in sheep provided with rumen fistulae, and catheters in the parotid duct, ureter and v. jugularis. The animals were supplied with different amounts of NaCl and water previously. $^{24}Na^+$ was used in the experiments.

The Na^+ concentration in the saliva decreased and the K^+ concentration increased considerably on the effect of insufficient NaCl supply. This resulted in a significant decrease of the Na^+/K^+ quotient. In the case of sufficient NaCl supply, $^{24}Na^+$ appeared in the saliva four times faster than in the case of insufficient NaCl supply.

Close connection was found between the secretion of saliva and urine flow. The decrease of the NaCl supply caused a diminution of the diuresis and saliva secretion as well as a decrease in the amount of Na^+ and K^+ excreted with the saliva and urine. The urine flow increased on the effect of NaCl loading but the saliva secretion did not change. Large amounts of Na^+ and K^+ were excreted in the urine. Thirst, through the consequent water overcharge changed the volume of rumen fluid and its osmotic pressure. This made an alteration in the diuresis and saliva production and in the amount of Na^+ and K^+ excreted with urine and saliva. In conclusion, the change of the osmotic pressure of the rumen fluid influences the water and electrolyte metabolism of ruminants rapidly and significantly.

The water and electrolyte content of the rumen has an important role in the regulation of water and electrolyte metabolism in the sheep (Wilson, 1968; Warner and Stancy, 1972*a*, *b*; Tomas et al., 1973; Wilson and Dudinski, 1973).

The ion concentration of the rumen fluid fluctuates within a wide range (English, 1966, 1967). Data on fluid exchange between the rumen content and the epithelium postulate that extensive water and electrolyte transport takes place across the rumen wall (Scott, 1967; Warner and Stancy, 1972*b*). Thus, a change in the osmotic pressure of the rumen fluid can modify the water space of the body (Engelhardt, 1970).

Diuresis is mainly responsible for the relative constancy of the water space as well as the distribution of electrolytes, although saliva secretion is

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also of great importance in ruminants (Coats and Wright, 1957; Potter, 1961, 1963; Stancy and Brook, 1964; Pickering, 1965).

In previous experiments (Juhász et al., 1977) on sheep, we studied the effect of water or salt loading, as well as the effect of water plus salt loading, on the extracellular (EC) space and on urine flow. According to our results, water loading caused a gradual enlargement of the EC space and developed a hypoosmotic hypervolaemia together with a hypoosmotic diuresis. Administration of salt did not alter the EC space essentially. Similarly, the diuresis changed only slightly, and a highly concentrated urine was excreted. The water-plus-salt loading was followed by absorption of both substances from the rumen. The Na^+ was absorbed slower than the water, therefore, the plasma Na^+ concentration did not change in the first hour, but the EC space increased. First an isoosmotic hypervolaemia, then a hyperosmotic hypervolaemia developed, the tubular re-absorption of Na^+ decreased and the diuresis increased. Thus, a relatively large amount of hyperosmotic urine was excreted.

In our present experiments, the mechanism of regulation of water and electrolyte metabolism was examined in sheep supplied previously with different quantities of salt or water and after an acute salt, or water, loading

Materials and methods

The experiments were carried out in 15 Merino sheep weighing 30 kg, provided with rumen fistulae and indwelling catheters in the parotid duct, ureter and jugular vein.

In the first and second series of the experiments, the animals were divided into three groups (A, B, C), in which the NaCl supply in the diet was plentiful (5.0% NaCl; groups A), sufficient (1.0% NaCl; B) and deficient (0.1%; C) for 4 weeks. Each group consisted of 5 animals. The diet contained 0.4 kg mixture of coarse meal (with vitamin and mineral premixes) and 0.5 kg hay. In the acute experiments, 300 ml water containing 17.1 g NaCl/kg body weight and 7.4 MBq $^{24}Na^+$ were given into the rumen.

In the third series of the experiments, animals were given water ad lib. (groups D and F) and others were deprived of water for 48 h (group E). The salt load was 17.1 mmol/kg body weight in groups D and F and 12.8 mmol/kg body weight in group E. In all cases, the NaCl was dissolved in 300 ml water, together with 7.4 MBq $^{24}Na^+$. The water load was 80 ml/kg body weight (group F), which also contained 7.4 MBq $^{24}Na^+$.

The change of Na^+ and K^+ concentrations, as well as the radioactivity, were determined in plasma, saliva, urine and rumen fluid. The rate of saliva secretion and diuresis was measured and registered automatically and each

was collected into fractions. In the acute experiments, which lasted 400 min, the values were determined in the first 40 min, before loading with salt or water (Juhász et al., 1975, 1976*a*, *b*; 1977).

Each value presented in Tables I–VI represents the mean of the results obtained during the experimental period. We did not present the values of all statistical calculations because only those results which showed the greatest changes were evaluated biologically.

Results

In the first series of experiments, we studied the Na^+ and K^+ concentrations of the plasma and the saliva, furthermore, the Na^+/K^+ quotient in the saliva on animals provided with different salt supplies (A, B and C) after an intraruminal NaCl overcharge. We measured the activity of the $^{24}Na^+$ (cpm) in the plasma absorbed from the rumen and its appearance in the saliva. We determined the space of time required by the activity of the saliva to exceed the activity of the plasma. In the first experiments, the mean concentrations of Na^+ in the plasma before the salt loading were between 141 and 149 mmol/l and those of K^+ between 4.3 and 4.8 mmol/l (Table I). After the salt loading, changes could be observed only in groups A and B.

Table I

The effect of different salt supplies on Na^+ and K^+ concentration of plasma and saliva in sheep before and after salt loading*

Groups	Salt supply	NaCl in fodder %		Plasma		Saliva		Na^+/K^+	Minutes**
				Na^+	K^+	Na^+	K^+		
				mmol/l					
A	plentiful	0.60	before	142	4.7	182	4.8	38	11
			after	157	4.6	178	4.3	41	
B	sufficient	0.45	before	141	4.8	155	5.4	28	42
			after	152	4.7	173	5.2	33	
C	deficient	0.20	before	149	4.3	116	12.6	9	40
			after	153	4.2	147	8.9	17	

* NaCl load was 1 g/kg body weight given into the rumen.
** The time being necessary for the radioactivity of saliva (cpm ^{24}Na) to exceed that of plasma.

In contrast to the plasma, the Na^+ concentration of the saliva before salt loading was different in the various groups and showed a positive correlation with the salt intake. The K^+ concentration in the saliva changed inversely to the Na^+ intake (A, 4.8; B, 5.4 and C, 12.6 mmol/l). After salt loading, the Na^+ concentration rose only in groups B and C. The K^+ concentration did not alter in groups A and B and decreased in C.

Before the salt load, the Na^+/K^+ ratio in the saliva had the following average values: 38 (group A), 28 (B) and 9 (C). After the salt load, the Na^+/K^+ ratio increased significantly in group C.

In group A, the time necessary for the radioactivity of saliva to exceed that of plasma was 11 min, whereas in groups B and C it averaged 41 min (Table I).

In the second series (Table II) the rates of saliva production and diuresis, furthermore, the rates of secretion and excretion of Na^+ and K^+ were studied before and after salt loading in animals with different salt supplies (A, B and C).

By plentiful salt supply, the rate of saliva secretion was 0.81 ml/min before, and 0.88 ml/min after, salt loading (group A). The rates of Na^+ and K^+ secretion did not change significantly. The rate of urine production increased after salt loading, as did Na^+ and K^+ excretion.

The saliva production rate of sheep with sufficient salt supply (group B) decreased. The rate of salivary Na^+ and K^+ secretion decreased from 97 to 66 and from 3.2 to 2.8 μmol/min, respectively. After salt loading the rate of diuresis and the Na^+ and K^+ excretion increased.

In the animals with deficient salt supplies (group C) the saliva secretion and the rate of Na^+ and K^+ secretion decreased. In contrast, the rate of

Table II

The effect of salt load* in sheep supplied previously with different quantity of NaCl

Groups	Saliva secretion		Salivary Na^+		Salivary K^+		Urinary volume		Urinary Na^+		Urinary K^+	
	before load	after load	before load	after load	before load	after load	before load	after load	before load	after load	before load	after load
	ml/min		μmol/min				ml/min		μmol/min			
A plentiful	0.81	0.88	161	159	3.8	3.9	0.79	1.24	44	184	243	305
B sufficient	0.70	0.45	97	66	3.2	2.8	0.66	0.84	36	140	42	104
C deficient	0.56	0.40	50	43	2.6	2.4	0.39	0.56	15	87	61	113

* NaCl load 1 g/kg body weight, given into the rumen.

diuresis, the Na^+ and the K^+ excretion increased after salt loading. As shown in Table II, the rate of Na^+ excretion in the urine and saliva was related to the level of Na^+ intake.

In the third series of experiments we studied the effect of salt loading on animals with water supply ad lib. (group D) and on thirsting animals (E).

We compare the results of these experiments with the values of experiments carried out on animals previously supplied with water ad lib. and after overcharged with water (group F). The Na^+ concentration and the volume of rumen fluid was increased by the salt overcharge (Table III). In both of groups D and E the Na^+ concentration became more than double and the rumen fluid increased to 1.3-fold in volume. The K^+ concentration did not

Table III

The change of Na^+, K^+ and fluid content in the rumen

Water supply and loading	Na^+	K^+	Rumen fluid litre
	mmol/l		
D) ad lib.	92	29	2.1
NaCl loading 1.0 g/kg bw*	241	28	2.7
E) thirst, 48 h	164	20	0.7
NaCl loading 0.75 g/kg bw*	431	18	1.0
F) ad lib.	100	26	1.8
water loading 80 ml/kg bw*	60	16	5.0

* body weight.

Table IV

The Na^+ and K^+ concentration of the plasma

Water supply and loading	Na^+	K^+	Minutes*
	mmol/l		
D) ad lib.	143	4.4	—
NaCl loading 1.0 g/kg bw	150	3.7	32
E) thirst, 48 h	156	4.1	—
NaCl loading 0.75 g/kg bw	158	4.1	14
F) ad lib.	153	4.3	—
water loading 80 ml/kg bw	148	3.7	38

* The time being necessary for the radioactivity of saliva (cpm ^{24}Na) to exceed the activity of the plasma.

change appreciably. On the effect of water overcharge, the volume of rumen fluid increased from 1.8 to 5.0 litres, and the Na^+ and K^+ concentration decreased from 100 to 60 and from 26 to 16 mmol/l, respectively.

In animals supplied previously with water ad lib. (groups D and F) the Na^+ concentration of the plasma after NaCl or water loading did not change significantly (Table IV). In both groups, the K^+ concentration of the plasma decreased. After thirst (group E), the Na^+ concentration showed a high value (156 mmol/l) which increased little on the effect of NaCl loading; the K^+ concentration of the plasma did not change.

After salt loading, the time being necessary for the activity of saliva to exceed the activity of the plasma was 32 min in the animals with water supply ad lib. (group D). In the case of salt loading after thirst (group E) this time was considerably shorter. After water overcharge in the animals with a previous water supply ad lib. (group F), the process was slower and lasted 38 min.

The Na^+ concentrations of the saliva were nearly identical in groups D, E and F before loading (Table V). On the effect of NaCl loading the concentrations increased slightly, whereas they decreased after water overcharge. In the same groups, the K^+ concentrations were 5.0 mmol/l before the overcharge and of thirsting animals (group E) 4.6 mmol/l, which in the saliva did not change after salt overcharge. In animals supplied with water ad lib. (groups D and F), both salt and water loading caused a decrease in the K^+ concentration of saliva.

Before the loading, the rate of saliva secretion was 1.41 ml/min in the thirsting animals (group E) and in the groups of water supply ad lib. (groups D and F) 0.86 and 0.57 ml/min. On the effect of NaCl loading, the values decreased particularly in thirsting animals. The water overcharge did not change the rate of saliva secretion.

Table V

The Na^+ and K^+ concentration and the secretion rate of saliva, as well as the rate of Na^+ and K^+ secretion

Water supply and loading	Na^+ mmol/l	K^+ mmol/l	Secretion rate ml/min	Secreted Na^+ μmol/min	Secreted K^+ μmol/min
D) ad lib.	176	5.1	0.86	152	4.4
NaCl loading 1 g/kg bw	181	4.7	0.68	122	3.2
E) thirst, 48 h	179	4.6	1.41	255	6.2
NaCl loading 0.75 g/kg bw	189	4.6	0.84	157	3.7
F) ad lib.	173	5.0	0.57	98	2.8
water loading 80 ml/kg bw	169	4.2	0.59	100	2.5

Table VI

The Na^+ and K^+ concentration of urine, the rate of urine flow and the rate of Na^+ and K^+ excretion

Water supply and loading	Na^+ mmol/l	K^+ mmol/l	Excretion rate ml/min	Excreted Na^+ μmol/min	Excreted K^+ μmol/min
D) ad lib.	107	218	1.07	114	233
NaCl loading 1 g/kg bw	211	62	1.42	300	88
E) thirst, 48 h	167	284	0.51	85	145
NaCl loading 0.75 g/kg bw	284	291	0.35	100	105
F) ad lib.	16	47	4.90	78	230
water loading 80 ml/kg bw	50	71	4.90	245	348

Before the loading, the rates of Na^+ and K^+ secretion were the highest in the thirsting animals (group E). In the animals supplied with water ad lib. the values were lower. On the effect of salt loading all values became considerably lower, especially those of thirsting animals in group E. In the case of water overcharge, the rate of Na^+ secretion did not change but that of the K^+ secretion decreased.

The Na^+ and K^+ concentrations of urine were higher for thirsting animals than for animals supplied with water ad lib. before the overcharge (Table VI). The NaCl, as well as the water overcharge, caused an increased Na^+ concentration in all cases. On the effect of salt loading, the K^+ concentration did not change in the urine after thirst, but became far lower after ad lib. water supply; it fell from 218 to 62 mmol/l. After overcharge with water, the K^+ concentration of urine increased from 47 to 71 mmol/l.

In group F the rate of urine flow was 4.9 ml/min before the water overcharge and did not change after it. In spite of this fact, the rates of Na^+ and K^+ excretions increased, due to the increase of their concentrations in the urine. The salt loading resulted in an increased rate of diuresis and of Na^+ excretion (from 114 to 300 μmol/min), and in a reduced K^+ excretion.

After thirst the rate of urine flow was very slow (0.51 ml/min) and on the effect of NaCl overcharge it became still slower (0.35 ml/min). The rate of Na^+ excretion increased only and the K^+ decreased slightly.

Discussion

The quantity of the Na^+ secreted in the ovine saliva is dependent on the total salt content of the sheep organism, which in turn depends on the NaCl intake in the fodder. The Na^+ secretion in the saliva increases with an

increasing intake of NaCl. In case of deficient saline supply (group C) the concentration of K^+ redoubles in the saliva. The time required by the cpm-number of $^{24}Na^+$ to become higher in the saliva than in the plasma is the shortest in animals with plentiful salt supply and in the control group; in the case of animals with deficient salt supply, the space of time is longer (Denton, 1956; Coats and Wright, 1957; Dobson et al., 1960; Warner and Stancy, 1972*a*; Scott, 1967).

Our experiments have shown that the saliva secretion and the urine flow change reciprocally during the regulation of water and electrolyte metabolism, mainly in the case of sufficient and deficient salt supply after NaCl or water loading. After load with saline, the Na^+ surplus is excreted only by the urine, while the Na^+ concentration and secretion in the saliva decreases. When the NaCl intake in the food is low the Na^+ and K^+ quantities secreted in the saliva and in the urine decrease (Potter, 1961, 1963 and 1968; Pickering, 1965; English, 1966, 1967; Anderson and Olsson, 1970). In all cases, the NaCl load increases the excreted quantities of Na^+ and K^+ in the urine but not in the saliva.

Comparing the results of rumen experiments with those of saliva and urine experiments we can state that the increase of the electrolyte (Na^+) concentration in the rumen content — in consequence of saline loading — causes a higher Na^+ and a lower K^+ concentration in the urine (Scott, 1967, 1975). In this case the rate of salivary secretion decreases and that of the urine flow increases. In this way, the urinary excretion of Na^+ becomes six times higher but the salivary secretion of Na^+ decreases (Scott, 1969).

Thirst causes an increase of Na^+ concentration in the rumen content; it redoubles the rate of salivary secretion and reduces the diuresis to its half. The secretion and excretion of Na^+ increases. In the case of thirst, more fluid and Na^+ returns to the rumen in the saliva, but less is excreted in the urine.

The intake of NaCl after thirst increases the Na^+ concentration of the rumen content (Wilson, 1966, 1967 and 1968; Warner and Stancy, 1972*b*; Wilson and Dudinski, 1973; Tomas et al., 1973). This results in an increase of Na^+ concentration of the urine. The parotid glands and the kidneys react to this with a decrease of the secretion rate and thus the urinary Na^+ excretion does not change.

The water overcharge increases the quantity of the rumen content and reduces the Na^+ and K^+ concentrations in it. At the same time, the Na^+ and K^+ concentrations increase in the urine and their excretion increases, too. Comparing our experimental results again we can establish that the intake of electrolytes and water, or the thirst do not change significantly the isotonia and isoionia, but they have a quick and significant effect on the electrolyte concentration and on the secretion of saliva and urine. The NaCl loading increases generally the Na^+ concentration in the urine, as well as the urine

flow, and it decreases the K^+ concentration in the urine and the secretion of the saliva. Thirst redoubles salivary secretion, but decreases the diuresis. Thirst is followed by an increase in the Na^+ secretion in the saliva and by an increase in Na^+ excretion in the urine. The salt loading after thirst decreases both the salivary secretion and the diuresis, as well as the Na^+ secretion in the saliva. The water overcharge increases the Na^+ concentration in the urine by the effect of rumen fluid increase and the excretion of Na^+ increases threefold in the urine.

The following conclusion can be drawn from our results: the change of the osmotic pressure in the rumen influences the water and electrolyte metabolism of the organism quickly and significantly.

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EFFECT OF SEXUAL STEROIDS ON LIPID AND LIPID-SOLUBLE VITAMIN TRANSPORT IN DOMESTIC FOWL

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The effects of sexual steroids (17-β-oestradiol alone and of its combination with progesterone and/or testosterone) on blood levels of total lipid, carotene and lipid-soluble vitamins A and E were investigated in non-laying hens. During a treatment of four weeks, the plasma levels of these compounds were measured weekly.

The total lipid content increased systematically in the groups treated with oestradiol, and oestradiol + progesterone. The carotene level showed non-significant variations. The vitamin E content of plasma increased significantly in the oestradiol-treated group at the end of the first week of treatment. The retinyl ester level showed a peak in the second week in the oestradiol-treated group. The changes in the retinol level were similar to those in vitamin E.

Fat-soluble vitamins are important factors in poultry nutrition. The requirement is difficult to determine because it depends on age, body mass and sexual maturity.

The plasma levels of sexual steroids fluctuate during the ovarian cycle. The effects of their changes extend to the vitamin transport as well. Effects of sexual steroids on vitamin A transport were first published more than 30 years ago. Chapman (1947) found that in immature chicken the vitamin A level in plasma increased after treatment with 17-β-oestradiol. Mézes and Bárdos (1981) reported that 17-β-oestradiol and progesterone increased plasma vitamin A level in immature chickens. Sexual steroids are involved in lipolytic activity as well (György, 1947). The excessive deposition of liver lipid is one of the side effects of oestrogens associated with the fatty-liver syndrome in laying hens (Akiba et al., 1982).

Materials and methods

The experimental birds were 65 weeks old Tetra-SL laying-type hybrids (Agricultural Combinate Bábolna) which had reached the end of their laying period. Non-laying hens were used to exclude the disturbing effects of endogenous changes in hormone production.

Commercial food of laying hens and drinking water were available ad libitum.

Intramuscular injections of 17-β-oestradiol propionate, testosterone phenylpropionate and progesterone (G. Richter Pharmaceutical Works, Budapest) were given daily, dissolved in sterilized sunflower oil.

The animals were grouped as follows: *Group 1*, 1.5 mg oestradiol kg^{-1} body mass; *Group 2*, 1.0 mg oestradiol + 0.5 mg progesterone kg^{-1} body mass; *Group 3*, 1.0 mg oestradiol + 0.5 mg testosterone kg^{-1} body mass; *Group 4*, sterilized sunflower oil.

The treatment lasted four weeks. Blood was taken weekly from wing vein into heparinized tubes.

The carotene content of plasma was measured according to McLaren (1967). The 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 retinyl ester content was expressed in retinyl acetate, which was used as standard. The total lipid content was measured turbidimetrically with Kunkel's reagent (MOM, 1971). Vitamin E was measured by the Emmerie-Engel reaction, using the method of Bieri (1975).

Student's t test was used for the statistical evaluation.

Results

The plasma levels of total lipid, carotene, retinol, retinyl and tocopherol are presented in Table I.

Significant differences were not found in the carotene level.

The vitamin A derivatives (retinol, retinyl esters) showed significant differences only between Group 1 and Group 4. The retinol content in Group 1 increased significantly after one week of treatment ($P < 0.001$) but dropped to the control level after the third week. No differences could be observed in Groups 2 and 3.

The retinyl ester level showed a peak at the end of the second week of treatment ($P < 0.001$) in Group 1, but then it dropped to the control level.

The vitamin E level showed a significant ($P < 0.001$) increase at the end of the first week, but decreased later.

The effective compound was 17-β-oestradiol at the dose of 1.5 mg kg^{-1} body mass.

The total lipid content increased in both of Groups 1 and 2, but these differences were significant only from the second week of treatment on.

Table I

Variations of plasma levels of lipid, carotene and vitamins A and E in non-laying hens treated with sexual steroids

Day of treatment	Group No.		Total lipid g/l	Carotene mg/l	Retinol μM/l	Retinyl μM/l	Tocopherol mM/l
7th	1	$\bar{x}$	56.48	1.07	2.87***	0.83***	2.50***
		s $\pm$	12.38	0.33	0.49	0.09	0.32
	2	$\bar{x}$	40.70	1.30	0.51	1.26	0.91
		s $\pm$	11.82	0.25	0.20	0.24	0.18
	3	$\bar{x}$	37.53	1.03	0.35	1.43	1.03
		s $\pm$	13.95	0.12	0.10	0.16	0.32
	4	$\bar{x}$	39.07	0.81	0.31	1.51	0.71
		s $\pm$	14.15	0.15	0.13	0.14	0.19
14th	1	$\bar{x}$	73.20**	1.33	1.59*	3.67***	2.41*
		s $\pm$	9.44	0.24	0.55	1.23	0.53
	2	$\bar{x}$	63.30*	0.76	0.88	0.96	0.78
		s $\pm$	6.07	0.31	0.29	0.17	0.23
	3	$\bar{x}$	57.56	0.60	0.68	1.10	0.77
		s $\pm$	12.11	0.17	0.29	0.37	0.26
	4	$\bar{x}$	44.70	0.88	0.56	0.81	1.18
		s $\pm$	10.35	0.34	0.21	0.13	0.50
21st	1	$\bar{x}$	94.00**	1.22	0.51	0.83	1.27
		s $\pm$	34.32	0.60	0.28	0.41	0.40
	2	$\bar{x}$	77.43*	0.73	0.50	1.23	0.66
		s $\pm$	22.39	0.32	0.27	0.24	0.16
	3	$\bar{x}$	80.02	0.95	0.31	1.21	0.72
		s $\pm$	37.75	0.40	0.10	0.53	0.18
	4	$\bar{x}$	49.52	0.66	0.55	0.99	0.93
		s $\pm$	3.73	0.33	0.24	0.18	0.19
28th	1	$\bar{x}$	113.52**	0.87	0.41	0.96	0.68
		s $\pm$	12.09	0.27	0.16	0.29	0.16
	2	$\bar{x}$	108.00**	1.07	0.51	1.26	0.74
		s $\pm$	14.45	0.46	0.26	0.60	0.19
	3	$\bar{x}$	93.60	1.07	0.53	1.05	0.72
		s $\pm$	19.93	0.30	0.21	0.23	0.11
	4	$\bar{x}$	63.47	1.03	0.41	1.19	0.60
		s $\pm$	13.27	0.38	0.17	0.13	0.23

In each group there were 10 animals.
* = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$.

Discussion

The present results agree well with the view that sexual steroids affect not only the sexual organs (Knox, 1956), but also the transport of minerals (Kemény et al., 1970), lipids (György, 1947) and lipid-soluble vitamins (Chap-

man, 1947; Mézes and Bárdos, 1981). The observable variations of vitamin A derivatives and vitamin E do not extend to the carotene transport. It seems that the effect of sexual steroids, mainly 17-β-oestradiol, is not a regulatory function on the intestinal absorption of vitamin A and carotene, but increases the mobilization of retinol from the liver and enhances the production of vitamin A from carotenes. This enhance was detected by the increased plasma retinyl ester content because this form of vitamin A is transported from the intestinal epithelial cells to the liver. At the same time, plasma carotene content did not change. The detected increase of vitamin E content shows that the effect of sexual steroids extends, besides vitamin A, to other fat-soluble compounds. This general non-specific effect was detected in the increase of total plasma lipid content.

The variations in vitamins A and E and total lipid content were different. The vitamin levels were significantly higher only in Group 1 while total lipid content increased in Group 2 as well. It means that the changes in plasma levels of vitamins A and E are dose-dependent of sexual steroids.

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DETERMINATION OF SOME NORMAL VALUES IN BLOOD SAMPLES OF CHICKEN, PHEASANT AND QUAIL EMBRYOS

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Serum alkaline phosphatase, Ca, inorganic P and haematocrit values and erythrocyte counts were determined in chicken, pheasant and quail embryos in the period preceding hatching. The simple blood sampling technique described and the use of appropriate diagnostic and biochemical micro-methods render possible the determination of several parameters from the blood or serum of embryos.

The 1311 determinations reported may serve as reference values, previously missing in the literature.

In toxicological studies on chemicals, among them pesticides, ecotoxicological investigations occupy an important place. These are aimed at detecting the toxic effects exerted on non-target animals. The last decade has seen an increase in the number of publications reporting embryotoxic and teratogenic effects of certain pesticides in avian (chicken, quail, pheasant) embryos. These, primarily Japanese, French and American, publications have almost exclusively dealt with the morphological aspects of the problem.

However, at the present time the studies on certain morphological and functional relationships are also considered important. By their help a more thorough understanding of the embryotoxic effects of the compounds and of the mechanism of these effects can be achieved. In addition, the potential perinatal (postnatal) hazards created by the materials examined can also be evaluated with more certainty.

The present paper attempts to be of help for those undertaking embryological and teratological studies on avian embryos, including studies on the changes occurring in certain blood and/or serum parameters.

Materials and methods

Fertile Japanese quail *(Coturnix coturnix japonica)*, chicken (Shaver Starcross 288) and pheasant *(Phasianus colchicus mongolicus et torquatus)* eggs were used. Blood samples were withdrawn from quail, chicken and pheasant

embryos on the 14th, 19th and 23rd day of incubation, respectively. After having removed the calcic egg shell from a circumscribed area, we passed the scissors' tip through the shell membrane, into the blood vessel network of the intact allantois. The appearing drop of blood originating from the a. umbilicalis was collected in heparinized glass capillaries (Várnagy, 1981). Haematocrit values and erythrocyte counts were determined in several series of tests (Bálint, 1963).

For biochemical studies, the cellular elements of the blood were separated from the plasma. The capillary part containing the sedimented cells was cut off, while the part containing the blood plasma was sealed airtight and stored until use.

For the determination of serum alkaline phosphatase (SAP) enzyme activity (Hausamen et al., 1967) a Satellite one-channel chemical analyser of discrete system and an LKB Ultrolab System 7400 Calculating Absorptiometer were used. The determination was made at 404 nm. Ca and inorganic P concentrations were determined (Richterich, 1971) by using a semi-automatic Spektromom 410 Modul S and the above LKB devices. Ca was determined at 570 and 584 nm, while inorganic P at 600 and 658 nm. La Roche 1028 and 1023, and Galenopharm reagents were used throughout the above determinations.

Results

A total of 1311 blood and serum samples obtained from embryos of the three avian species were examined for three parameters. Detailed data are given in Tables I and II. The SAP activities of the quail and the chicken embryo were similar, while for pheasant embryos a slightly higher mean SAP value was obtained. The mean Ca values were almost identical for pheasant and quail embryos, while in chicken embryos a somewhat lower value was found. Chicken and pheasant embryos showed nearly identical, while quail embryos had slightly higher, inorganic P values.

Table I

Some serum parameters of chicken, pheasant and quail embryos ($\bar{x} \pm$ S.D.)

Parameter	Chicken	Pheasant	Quail
SAP (U/l)	756 ± 217 n = 59	931 ± 169 n = 101	734 ± 180 n = 61
Ca (mmol/l)	1.88 ± 0.48 n = 71	2.67 ± 0.44 n = 102	2.66 ± 0.56 n = 61
Inorganic P (mmol/l)	2.44 ± 0.67 n = 76	2.34 ± 0.65 n = 104	3.01 ± 0.87 n = 61

n = number of samples

Table II

Some blood parameters of chicken, pheasant and quail embryos ($\bar{x} \pm$ S.D.)

Parameter	Chicken	Pheasant	Quail
Haematocrit value	0.2870 ± 0.0110 $n = 148$	0.3006 ± 0.0290 $n = 180$	0.2198 ± 0.0485 $n = 186$
Erythrocyte count (10^{12}/l)	2.92 ± 0.32 $n = 26$	2.39 ± 0.66 $n = 27$	1.45 ± 0.37 $n = 48$

n = number of samples

Haematocrit values were the lowest in quail, and the highest in pheasant embryos, chicken embryos showed an intermediate value. Lowest and highest mean erythrocyte counts were found in quail and chicken embryos, respectively.

Discussion

The blood sampling technique described above was found to be reliable and reproducible. In the period preceding hatch-out the blood vessels of the chicken embryo are well-developed (Kovács and Fehér, 1966; Romanoff, 1960) and the volume of its blood is considerable. The allantoic membrane of quail and pheasant embryos also contains well-developed blood vessels (Várnagy, 1981), from which blood can easily be withdrawn by puncture at the end of the incubation.

Examination of the embryonic skeleton occupies an important position in teratological studies. The Ca content of the chicken embryo as a function of incubation time was studied by Romanoff (1967). At the time chosen by us, both the Ca and the inorganic P concentrations of the serum can easily be determined. Since the osteoblasts having an important role in bone development are also involved in the synthesis of SAP, the SAP value can be considered indicative of the actual osteoblast activity.

From blood and serum collected in heparinized glass capillaries, certain serum parameters can be determined by micro-methods using automatic analysers (Satellite, LKB) (Bartalits et al., 1978; Toshijuki, 1979). The amount of blood obtainable by this sampling procedure varies between species; usually it is between 100 and 500 μl, a volume sufficient for the determination of several parameters.

By describing the above blood sampling technique, determination methods and experimental data, the present paper attempts to be of use for

research workers interested in avian embryology, particularly in teratological problems. It also supplies some previously missing haematologic and biochemical data.

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EFFECT OF PRESLAUGHTER FACTORS ON SERUM CREATINE PHOSPHOKINASE AND LACTATE DEHYDROGENASE ENZYME ACTIVITIES IN PIGS

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Serum creatine phosphokinase (CPK) and lactate dehydrogenase (LDH) enzyme activities were studied in pigs slaughtered either immediately after their arrival at the abattoir following careful transportation to a distance of 35 km, or after having been exercised on a 350 m long course. The studies were extended to pigs transported in the summer, autumn and winter periods, and both hybrid pigs and those of a simple crossing were studied.

The season and the ambient temperature were found to influence the serum parameters studied. The lowest (i.e. the most advantageous) enzyme activities were observed in pigs transported in October, while the highest (least advantageous) values in those transported in January and February.

The adverse effect manifested itself not only in altered average values within the groups but also in the particularly high enzyme activities found in some individuals.

The exercise between transportation and slaughtering, as an additional stress, failed to induce demonstrable changes in the parameters studied.

No breed differences were found in the parameters of blood withdrawn from pigs in the same season and under similar circumstances. Thus, no differences in stress susceptibility could be established on this basis.

The increased stress susceptibility of pig breeds selected for high meat production and growth rate, the occurrence of porcine stress syndrome (PSS) and of pale, soft, exudative (PSE) and dark, firm, dry (DFD) meat cause more and more trouble for both producers and pork industry.

Animals of increased stress susceptibility hardly tolerate moderate physical exercise, transportation, unfamiliar environmental factors, and they may respond to a moderate stress with death. Their muscles show enhanced anaerobic metabolic activity, the amount of lactic acid and the body temperature increase, and muscle spasms occur. Several authors (Bickhardt et al., 1977; Ludvigsen, 1980; Ollivier et al., 1975; Steinhardt et al., 1975) suggest that the low stress tolerance is correlated with the decreased capacity for aerobic energy production, while the temperature rise is primarily due to the aerobic metabolism of muscles, and only to a lesser extent to the anaerobic processes (Lister et al., 1976).

Stress susceptibility is of hereditary nature (Hwang et al., 1978; Ollivier et al., 1975; Schmidt et al., 1970; Schmitten and Schepers, 1979; Schulman, 1980; Webb, 1980). Factors other than genetic have also been incriminated,

e.g. weather conditions on the day of slaughter and on the day preceding it, have been shown to influence meat quality in 10%. The distance of transportation and the effects exerted on the animal at the abattoir influence the frequency of PSE/DFD meat in 15–17%. If the pigs are kept waiting at the abattoir too long before slaughter, this results in the evolution of DFD meat. On the other hand, a too short preslaughter waiting period leads to PSE meat.

In addition to the above factors, the managemental and keeping technology is also known to have a significant influence on the development of stress susceptibility and substandard meat quality (Szilágyi and B. Kovács, 1979; Szilágyi and Takács, 1981; Szilágyi et al., 1981).

The present investigations were undertaken to determine the influence of transportation under different weather conditions and at different temperatures, and of the additional physical exercise performed immediately after transportation and before slaughter, on certain serum parameters and stress susceptibility of the two pig breeds studied.

Materials and methods

A total of 174 hybrid and Hungarian Large White × Dutch Landrace F_1 pigs were used. The pigs were transported to the abattoir (to a distance of 35 km) by motor trucks in July, October or January. Half of the animals

Table I

CPK and LDH enzyme activities (U/l) in sera of hybrid and crossbred pigs exposed to different preslaughter stress conditions

	Date of transportation	Temperature at transportation °C	Number of pigs	CPK U/l	LDH U/l
Hybrid pigs	July 10	17	18	341 ± 102	346 ± 68
slaughtered after exercise	October 9	4	14	183 ± 34	347 ± 81
	January 22	−8	18	612 ± 416	463 ± 98
slaughtered immediately	July 24	17	16	285 ± 63	310 ± 65
after arrival	October 23	−4	12	371 ± 95	384 ± 79
Crossbred pigs	August 5	19	16	320 ± 98	342 ± 65
slaughtered after exercise	October 2	2	14	238 ± 103	295 ± 54
	January 29	−10	18	560 ± 327	456 ± 88
slaughtered immediately	July 17	15	16	329 ± 88	390 ± 44
after arrival	October 16	7	16	243 ± 45	269 ± 70
	February 12	−6	16	449 ± 348	429 ± 91

were slaughtered immediately after arrival at the abattoir, while the remaining pigs after having been exercised on a distance of 350 m.

Blood samples were taken at slaughtering. The serum activities of creatine phosphokinase (CPK, EC 2.7.3.2) and lactate dehydrogenase (LDH, EC 1.1.1.27) were determined. Enzyme activity was determined by the Boehringer test collection (CPK activity was determined by an "activated monotest", while LDH activity by an "optimized standard method"). The results were evaluated by analysis of variance.

Results

CPK and LDH enzyme activities found in the serum of pigs exposed to different preslaughter effects are shown in Table I, while the results obtained by analysis of variance in Tables II and III.

The lowest enzyme activities were usually observed in sera of pigs transported in October, while both enzymes consistently showed the highest activity in sera of pigs transported in the winter months.

Table II

CPK enzyme activity (U/l) in sera of hybrid and crossbred pigs exposed to different preslaughter effects

Treatment	Season			Average
	summer	autumn	winter	
Hybrid pigs				
exercised	341	183	612	379
not exercised	285	371	—	328
Crossbred pigs				
exercised	320	238	560	373
not exercised	329	243	449	340
Average	319	259	540	
Mean values ($\bar{x}$) for				
Hybrid pigs	313	277	612	354
Crossbred pigs	324	240	504	356
Exercised pigs	330	210	586	375***
Non-exercised pigs	307	307	449	334

The difference between averages for seasons is 102 (S.D. = 1%); the value of interrelationship between treatment and season is 66 (S.D. = 10%).
*** = $P < 0.001$.

Table III

LDH enzyme activity (U/l) in sera of hybrid and crossbred pigs exposed to different preslaughter effects

Treatment	Season			Average
	summer	autumn	winter	
Hybrid pigs				
exercised	346	347	463	385
not exercised	310	384	—	347
Crossbred pigs				
exercised	342	295	456	365
not exercised	390	269	428	362
Average	347	324	449	
Mean values ($\bar{x}$) for				
Hybrid pigs	328	365	463	366
Crossbred pigs	366	282	442	363
Exercised pigs	344	321	460	370
Non-exercised pigs	350	326	428	368

The difference between the averages for seasons is 33 (S.D. = 1%).

The enzyme activities of hybrid and crossbred swine transported in the same season were nearly the same. No considerable differences were demonstrable between serum parameters of pigs slaughtered immediately after transportation and of those subjected to exercise after transportation and prior to slaughter.

Discussion

Due to their characteristic function, muscles contain a number of enzymes catalysing processes associated with energy generation or deposition, or oxidation-reduction. From muscle cells suffering damages of various origin, these enzymes get into the extracellular space, thus resulting in altered enzyme activities of the blood serum (Kolb, 1979).

Therefore, rises in serum enzyme activities primarily indicate muscle damage and stress susceptibility (Addis et al., 1974; Bickhardt et al., 1977; Szilágyi and Felkai, 1978). Increased muscle work also leads to a rise in the activity of these enzymes.

In the present work, the highest CPK and LDH activities were found

in pigs transported in the winter months. The ambient temperature averaged —8 °C at the time of transportation. Temperature has been reported to have a role as a stress factor (Reddy et al., 1971). The unfavourable effect of transportation in the summer months, at higher temperatures (17 °C) is also expressed in the average values of the enzymes studied. On the other hand, in January and February the effect of temperature is exerted in a different manner. At low temperatures the animals obviously feel cold, and the resulting shivering and enhanced muscular exertion lead to increased enzyme activities ($P < 0.01$). Relatively high CPK and LDH activities were found in sera of pigs transported on an extraordinarily cold autumnal day, October 23 (mean temperature for the time of transportation, —4 °C). The high values of standard deviation indicated that some pigs were particularly sensitive to cold stress.

In the CPK activity the interrelationship of macroclimate and exercise ($P < 0.1$) was expressed. In addition to the seasonal effect, the exercise was demonstrated to enhance serum enzyme activities (at the level of $P < 0.01$). Upon exposure of pigs to cold, LDH activity increased significantly ($P < 0.01$), as compared to the other two seasons. This was also related to enhanced heat production and muscular trembling caused by transportation at low temperatures.

In pigs subjected to load, metabolic activity and flux of substrates through cellular membranes are enhanced due to hormonal influence. The flow of enzymes also corresponds to the concentration decrease (Lengerken and Pfeiffer, 1977). The altered cellular energy balance due to load results in increased permeability of the cell membranes. Therefore, certain authors (Donath and Strauzenberg, 1975) are of the opinion that changes in plasma enzyme activities fail to give primary information on the actual status of metabolism; these changes indicate only structural alterations due to the effect of altered intracellular energy balance. In such cases, primarily enzymes of skeletal muscle, heart and liver origin show concentration rises.

The effect of the additional physical load, the exercise of animals after arrival at the abattoir and prior to slaughter, on serum parameters could not be determined consistently. Although several authors (Berschneider and Wilsdorf, 1976; Jørgensen and Hyldgaard-Jensen, 1975; Laiblin and Jaeschke, 1979; Löwe et al., 1977) reported increased CPK and LDH activities upon intensive muscle work, e.g. exercise, these results were obtained in animals that had been forced to make only minimal muscular exertion. In the present case, the pigs were transported by motor trucks, thus, from the viewpoint of enzyme activity, forced moving did not prove to be an excessive physical load, as compared with the stress represented by loading and unloading.

Serum parameters of hybrid and crossbred pigs transported and slaughtered under similar conditions gave consistent values.

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A. I. BULLS' SEMINAL PLASMA ENZYME ACTIVITIES AS INDICATORS OF SPERMATOZOA MOTILITY, FERTILITY AND FREEZABILITY

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Forty-four artificial insemination (A. I.) bulls of the Hungarian Fleckvieh breed were divided into two groups according to their reproductive records (semen picture, freezability and fertility). Seminal plasma glutamic-oxalacetic transaminase (GOT), glutamic-pyruvic transaminase (GPT) and hyaluronidase enzyme activities were examined and correlated with the semen picture. GOT and GPT activities were measured, while the hyaluronidase enzyme was estimated by an immunodiffusion technique with rabbit anti-hyaluronidase serum. All the three enzyme values were higher in the seminal plasma of the bulls of poor reproductive quality than in that of the excellent bulls.

Different theories have been advanced concerning the site of action of the seminal plasma, which was considered a poor medium for storage of spermatozoa. The spermatozoa of most animal species survive only for a short time in it (Salisbury and Lodge, 1962). This is remarkable, for the composition of bull semen and seminal plasma was thought to provide valuable information for investigators interested in preservation methods. Certain parameters have been studied comparatively. The GOT content in epididymal and seminal plasma showed direct correlation to the sperm concentration (Einarsson, 1971). This is of special interest, for GOT being an enzyme of intracellular origin, its level in the plasma must reflect the degree of the injury in the spermatozoa. The GOT enzyme level was found to be lower in the seminal plasma than in the sperm, while the GPT enzyme activity is somewhat higher in the seminal plasma than in the sperm. No real difference was found between the cauda epididymidis and the ejaculate (Einarsson et al., 1970). In earlier investigations into the role of the testicular hyaluronidase, the dispersal activity of this enzyme prompted several workers to postulate that denudation of the ovum was a necessary antecedent to the penetration of the spermatozoon into it. On this basis, other workers reported that either direct or indirect addition of hyaluronidase preparation to the semen promoted fertility in artificial insemination. It has been found that in animals, such as the rabbit, in which the cumulus cell mass is not dispersed before fertilization, hyaluronidase released by spermatozoa may aid penetration through this layer (Austin, 1960).

In animals, e.g. the sheep, in which the cumulus cells are dispersed before fertilization, there is hardly any evidence for the presence of hyaluronidase in the acrosome.

Foulkes and Watson (1975) showed that sperm damage is due to loss of acrosomal integrity. The increase in hyaluronidase activity in the seminal plasma from the diluted semen after freezing and thawing suggests that acrosomal damage had occurred at this stage and it might be inferred that those samples demonstrating a larger increase in the total hyaluronidase activity would exhibit lower fertility. The purpose of this investigation was to study the normal enzyme levels of the seminal plasma, in relation to semen quality. We assumed that the enzyme levels might provide parameters useful in choosing semen samples for storage.

Materials and methods

Semen samples were collected from 44 A.I. bull sires of the Hungarian Fleckvieh breed. The animals were used as semen donors at the state farm of the Artificial Insemination Centre of Budapest. The animals were divided into two groups on the basis of their reproductive record and, after 10 weeks, on the basis of their clinical examination.

Group 1 included 20 bulls of excellent semen quality, characters and deep-freezing ability. Group 2 included 24 bulls of poor semen quality. Semen samples were considered to be of poor quality if their clinical parameters showed values unacceptable for using the samples as fresh or stored spermatozoa. This means samples of low fresh motility, low sperm concentration, high incidence of abnormal spermatozoa, low sperm motility after deep-freezing and thawing. The bulls of such semen samples displayed low fertility. Seminal plasma samples were separated by centrifuging the bulls' semen samples at 3000 *g* for 10 min and were immediately used for enzyme assays. GOT and GPT enzymes were measured with colorimetric methods at 546 nm. Hyaluronidase enzyme was estimated by an immunodiffusion method.

Composition of the agarose medium:

Phosphate buffer of pH 6.0	50 mM
Sodium chloride	0.25 M
Agarose gel	1%

Hyaluronic acid, 1 mg/ml was added to the medium after the latter had been boiled, and cooled to 55 °C.

To obtain monospecific antiserum to bovine hyaluronidase, we employed an improved procedure. From several plates in which rabbit antiserum formed

precipitate with bovine hyaluronidase, the precipitate lines were cut out and emulsified with complete Freund's adjuvant. Rabbits were immunized with this emulsion. The antiserum obtained from rabbits was better than the original one.

The immunodiffusion reaction was carried out on 76×51 mm slides. Three ml of the agarose gel and 50 μl antiserum were used on each slide. Each sample was examined in three wells, viz., undiluted, and diluted 1 : 2 and 1 : 4. In a further well, a reference sample and in another a pool of seminal plasma samples collected from normal bulls were placed (standard reaction). The slides were placed in a polystyrene box containing cotton saturated with water, and kept at room temperature for 24 h. The diameters expressed in millimetre were compared with the corresponding standard reaction, which was 11.5, 9.4 and 7.8 mm on the average (sum: 28.7) for the 1 : 1, 1 : 2 and 1 : 4 dilutions of the standard pool, respectively. The data within both groups were subjected to analysis of variance.

Results

All three enzymes determined in the fresh seminal plasma significantly differed ($P < 0.001$) between the two groups of semen. The mean $\pm$S.D. for the GOT and GPT values were 273.75 ± 59.55 U/l and 64.50 ± 22.86 U/l, respectively, for the group of good semen quality (group 1) and 399.16 ± 97.68 U/l and 149.33 ± 95.32 U/l for the group of poor semen quality (group 2), respectively (Tables I and II).

The hyaluronidase immunodiffusion reactions extended to 12, 10, and 8 mm diameter (sum, 30 mm) in group 1 and 16, 12 and 9 mm (sum, 37 mm) in group 2. Expressed the sums in per cent of the sum of the standard reactions (28.7 mm), the hyaluronidase reaction was 104% for group 1 and 129% for group 2. The group of excellent semen quality showed highly significantly better results than the group of bad semen quality (Table III).

Discussion

Bulls' semen and seminal plasma analysis is of major importance for many scientists in their studies on male reproductive processes and in evaluation of spermatozoa before using them for storage and artificial insemination purpose. It must be emphasized that seminal plasma analysis can give valuable information not only on semen quality, but also on the secretory function of the accessory genital glands. Investigations into the enzymes examined in the present study have been reported by several authors and the results have been related to sperm motility (Austin, 1960). The present results are directly

Table I

GOT, GPT and hyaluronidase enzyme activities in groups of bulls with good and bad semen

Bulls of good semen (n = 20)					Bulls of bad semen (n = 24)				
Hyaluronidase*			GOT (U/l)	GPT (U/l)	Hyaluronidase*			GOT (U/l)	GPT (U/l)
1/1	1/2	1/4			1/1	1/2	1/4		
dilution					dilution				
12	10	8	150	15	16	11	8	350	50
14	11	9	250	56	15	12	9	370	69
12	10	8	240	70	14	12	9	340	180
11	9	7	290	60	15	11	8	480	125
12	10	9	185	50	16	11	8	370	80
12	9	8	250	50	15	12	10	450	285
13	11	9	320	120	16	12	8	740	500
14	11	9	310	45	17	13	11	350	160
11	9	7	150	45	15	11	9	380	100
12	10	8	310	50	16	12	10	210	150
11	9	8	370	80	16	11	10	335	245
13	11	8	300	84	16	12	9	490	115
11	9	7	300	95	16	12	8	390	140
12	10	8	285	40	15	12	9	300	200
11	10	8	350	85	16	12	10	370	105
12	10	8	295	75	18	13	10	350	75
11	9	7	350	70	16	12	8	410	130
14	11	9	250	80	18	13	9	335	130
10	10	7	290	60	17	13	9	420	240
12	11	8	250	55	17	13	9	385	95
					16	12	8	445	80
					18	13	10	480	120
					15	11	8	350	100
					15	12	9	480	114
$\bar{x}$ 12	10	8	273.75	64.50	16	12	9	399.16	149.33
SD ±1.55	1.27	1.08	59.55	22.86	2.01	1.12	1.63	97.68	95.32

* Hyaluronidase values are expressed as the diameter of the immunodiffusion ring (mm).

comparable with sperm viability and storability. Besides, it should be noted that the fertility and freezability of sperm was found to be affected by the great loss of these enzymes from sperm head, which takes place during preparation for use in artificial insemination.

Table II

GOT, GPT and hyaluronidase enzyme activities in seminal plasma, and clinical parameters of bull semen

Parameters	Bull semen quality	
	Group of excellent bulls	Group of poor bulls
Seminal plasma enzymes studied		
GOT (U/l)	273.75	399.16
GPT (U/l)	64.50	149.33
Hyaluronidase (mm)	12/10/8	16/12/9
Laboratory clinical parameters (mean ±S.D.)		
pH	6.4 ± 0.13	6.3 ± 0.17
Volume (ml)	6.0 ± 3.8	4.0 ± 0.58
Sperm count ($\times 10^9$/ml)	1.4 ± 0.17	0.9 ± 0.42
Abnormal sperm (%)	21.0 ± 12.6	33.0 ± 10.4
Motility (%)	68.0 ± 6.4	59.0 ± 13.1
Motility after rethawing (%)	43.0 ± 8.7	38.0 ± 4.5
Fertility (%)	49.0 ± 8.1	38.0 ± 14.5

Table III

Analysis of variance of semen characteristics between groups of bulls with excellent and poor semen quality

Treatment	pH	Volume ml	Concentration $\times 10^9$/ml	Abnormal sperm %	Motility %	Motility after 9 min deep-freezing and thawing	Fertility %
Between Groups 1 and 2	N.S.	N.S.	$P < 0.001$	$P < 0.001$	$P < 0.001$	$P < 0.001$	$P < 0.001$

N. S. = not significant; $P < 0.001$ = highly significant.

The results of William (1972) are in good accordance with our present results that hyaluronidase is structurally inactivated by being bound in or under the plasma membrane. A chemical agent or an event, such as sperm death, that labilizes membranes, releases enzymes into the surrounding medium (seminal plasma). The present results agree well with the results of Foulkes and Watson, 1975, Zaneveld et al., 1973, Erickson and Krzanowska, 1974, Gould and Bernstein, 1973, that sperm damage means loss of acrosomal integrity. The increase in the concentration of hyaluronidase and other substances in the seminal plasma indicates that acrosomal damage had occurred

at this stage. On the other hand, high level of these enzymes in seminal plasma can be considered an indicator of damaged sperm cells. Morton (1975) showed that hyaluronidase and proteolytic enzymes are the best-characterized penetration enzymes and it seemed possible that strain-specific variations in their activities might be related to the different fertility.

The experiments described above are an extension of the study by Foulkes and Watson (1975) and were designed to develop a new simple technique. Since many procedures for processing liquid semen have been utilized, in part for frozen semen without further testing sperm motility, acrosome morphology, oxygen utilization and enzyme activities before cooling, after cooling to 5 °C, before freezing and after freezing and thawing, we considered necessary to measure all these parameters. We conclude that the determination of the above enzymes in the seminal plasma provides far better standard parameters for evaluating A.I. bulls' spermatozoa and to detect the delicate sperm which are not suitable for storage and deep-freezing processes, parameters which are valuable in male fertility control.

The entire and accurate system of A.I. bulls' semen handling in straws and deep-freezing processes should be tested in field trials in order to ensure optimum fertility with this method. The author strongly suggests that these enzymes are needed for moving of the sperm cell and fertilizing the ovum, therefore, must be checked regularly especially in semen samples to be used for artificial insemination and storage. The values of these enzymes can inform us about the condition of sperm cells more clearly than a lot of parameters and factors used for controlling and evaluating semen. An increased activity of these enzymes in the surrounding medium means that *(i)* the sperm has lost its fertilizing ability, *(ii)* the sperm has become abnormal, *(iii)* the sperm has been damaged or lost its acrosomal integrity, *(iv)* the sperm ultrastructure has changed, *(v)* the sperm has died, or *(vi)* the sperm has lost its normal shape, morphology and contents. All these explain the importance of determining these enzymes in the evaluation of semen for storage and artificial insemination.

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MORPHOLOGICAL ALTERATIONS OF BULL SPERMATOZOA DURING FREEZING AND THAWING

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A deeper understanding of the physiology, pathophysiology and morphology of the male genital tract and spermatozoal structure can give us a better chance to diagnose and treat dysfunctions in this organ system and also a basis for rational research on new means to control male fertility.

One hundred A. I. bull sires of Hungarian Fleckvieh breed were used in this study. The changes of the acrosome picture were scored using the method of acrosome score types 0–3 in different stages, viz. (A) fresh semen without treatment, (B) semen diluted and equilibrated for 3½ h, (C) semen frozen at —196 °C and thawed at 46 °C for 1 h, and (D) semen frozen at —196 °C, stored in liquid nitrogen for 24 h and rethawed. The number of defective spermatozoa and acrosome disruption increased under the freezing conditions. There was a positive correlation between acrosome picture and sperm motility, motility after thawing and fertility. Acrosomal structures can be estimated by the simple and rapid acrosome score technique which can be used in routine semen evaluation, sperm behaviour, sperm freezability and fertility control as a further criterion and excellent light-microscopic parameter. This parameter is accurate and sensitive enough for controlling the normality of the cells.

Frozen semen of many animal species has proved to be less fertile than fresh semen even if 60 to 70 per cent of the spermatozoa may remain motile after freezing and thawing. It seemed, therefore, to be justified looking for additional parameters, first of all morphological in nature.

Moench (1931), Williams (1964), Eliasson and Treichl (1971), Eliasson (1975) and Watson (1975) established that the morphology of spermatozoa is an important parameter in the routine evaluation of male fertility. A few reports have mentioned sterility of bulls which in their sperm population showed a dominant morphological defect located mainly or exclusively in the sperm tail (Blom and Birch-Andersen, 1966).

Coulter and Foote (1975) concluded that the ability of sperm to survive at body temperature after freezing and thawing decreases from stage to stage of semen processing. Acrosome damage increased at each successive stage. These observations prompted researchers interested in artificial insemination projects to pay attention to light- and electron-microscopic changes of the sperm head (Veres and Öcsényi, 1968*a*, *b*, *c*, 1973; Veres et al., 1972; Öcsényi and Veres, 1968).

Materials and methods

Bulls. The semen donors were 100 A. I. bull sires of Hungarian Fleckvieh breed used in the Artificial Insemination Centre. The parameters of the semen quality of the bulls were recorded (pH, volume, sperm count, abnormalities, progressive motility, motility after thawing, and fertility). The investigations of sperm head and acrosome in the present study included the changes of the acrosome picture in different steps of deep-freezing.

Samples. Semen samples were collected by the means of artificial vagina. All clinical parameters of semen were examined and then every ejaculate was diluted according to its spermatozoa concentration ($\times 10^9$/ml) in buffered egg yolk–glucose diluent, by using routine procedures of semen deep-freezing as performed at the Artificial Insemination Centre, Gödöllő.

The diluted semen was equilibrated for 3 1/2 h to 4 °C and then sealed in artificial straws (0.25 ml) by an automatic machine. The straws were frozen to —120 °C (9 min) in liquid nitrogen and transferred for storage at —196 °C into liquid nitrogen containers. From each diluted semen some samples (straws) were chosen and thawed in a water bath of 46 °C. Spermatozoa motility was recorded immediately and after 1 h.

Acrosomal structures were investigated during the chilling and deep-freezing procedures as follows. A drop of diluted semen was smeared on a slide and dried in a current of warm air. From every bull semen sample, 4 smears were prepared: (A) a drop of diluted fresh semen; (B) a drop of diluted semen after being equilibrated for 3 1/2 h; (C) a drop of diluted semen frozen at —196 °C for 9 min and then thawed at 46 °C for 1 h; and (D) a drop of diluted semen frozen at —196 °C, stored in liquid nitrogen for 24 h and thawed. The smears were immersed in buffered 10% formalin for 20 min and then washed in running tap-water for 30 min. Subsequently, the slide smears were transferred into buffered 10% tannic acid for 5 min and washed once more in running tap-water for 10 min. Finally, the slide smears were dried and then immersed in buffered 3% Giemsa solution for 100 min, rinsed briefly in distilled water and dried. Stained spermatozoa were examined randomly with a light microscope (see Legends to the figures). A subjective scoring (Watson, 1975) was used for evaluating the progressive disruption of acrosomes. The score ranged from 0 (undamaged acrosomes) to 3 (severely damaged or completely lost acrosomes) (Figs 1–4). The percentage of normal acrosomes was calculated for 10 spermatozoa selected at random on each slide. The mean score for 10 spermatozoa on each slide was used in the analysis of the results.

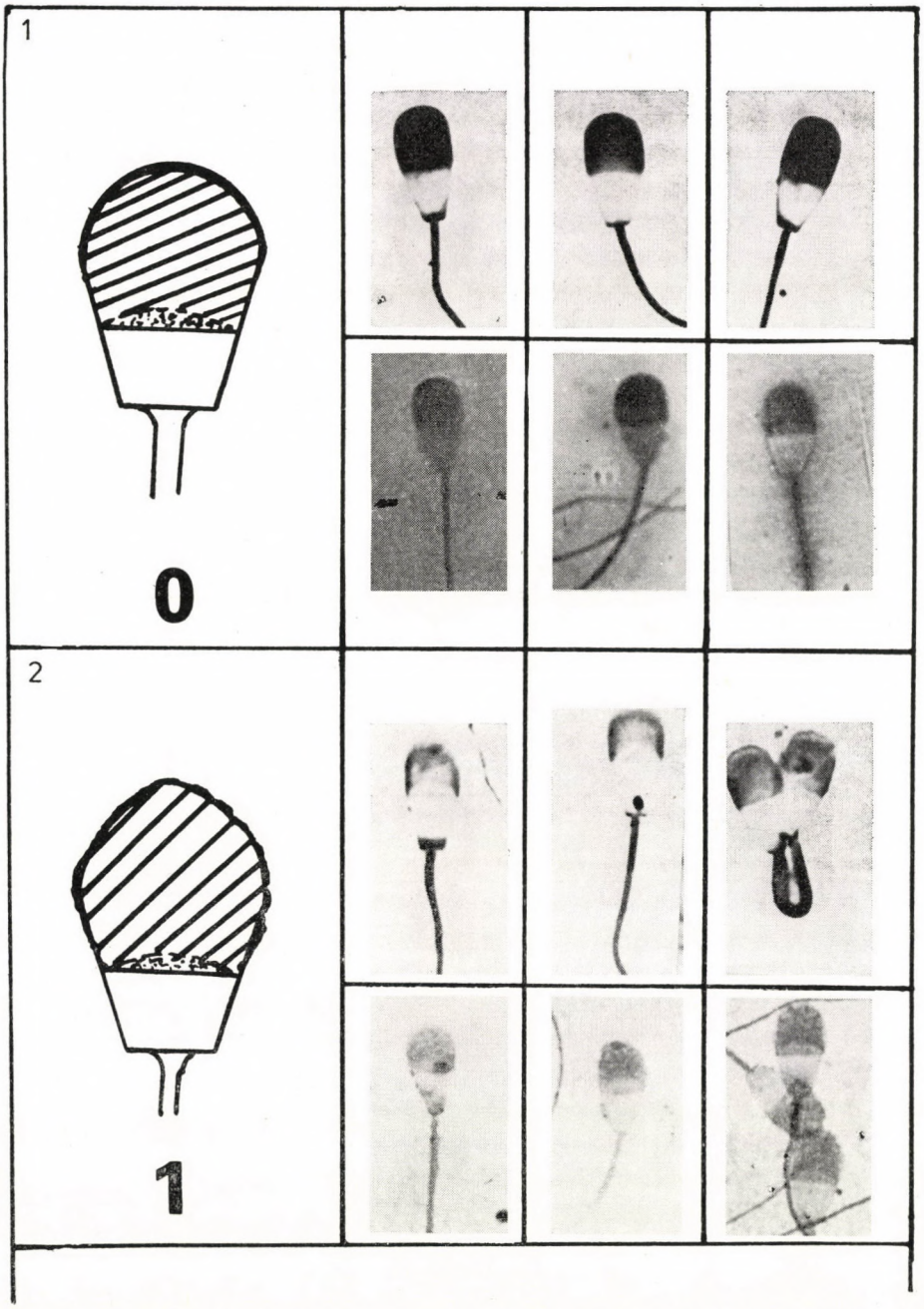

Fig. 1. Line drawing of sperm acrosome picture (score 0, normal acrosome). Light micrographs of A. I. bulls' spermatozoa acrosomes (normal acrosome, score 0). Phase contrast ×1350

Fig. 2. Line drawing of sperm acrosome picture (score 1, damaged acrosome). Light micrographs of A. I. bulls' spermatozoa acrosomes (score 1). Phase contrast ×1350

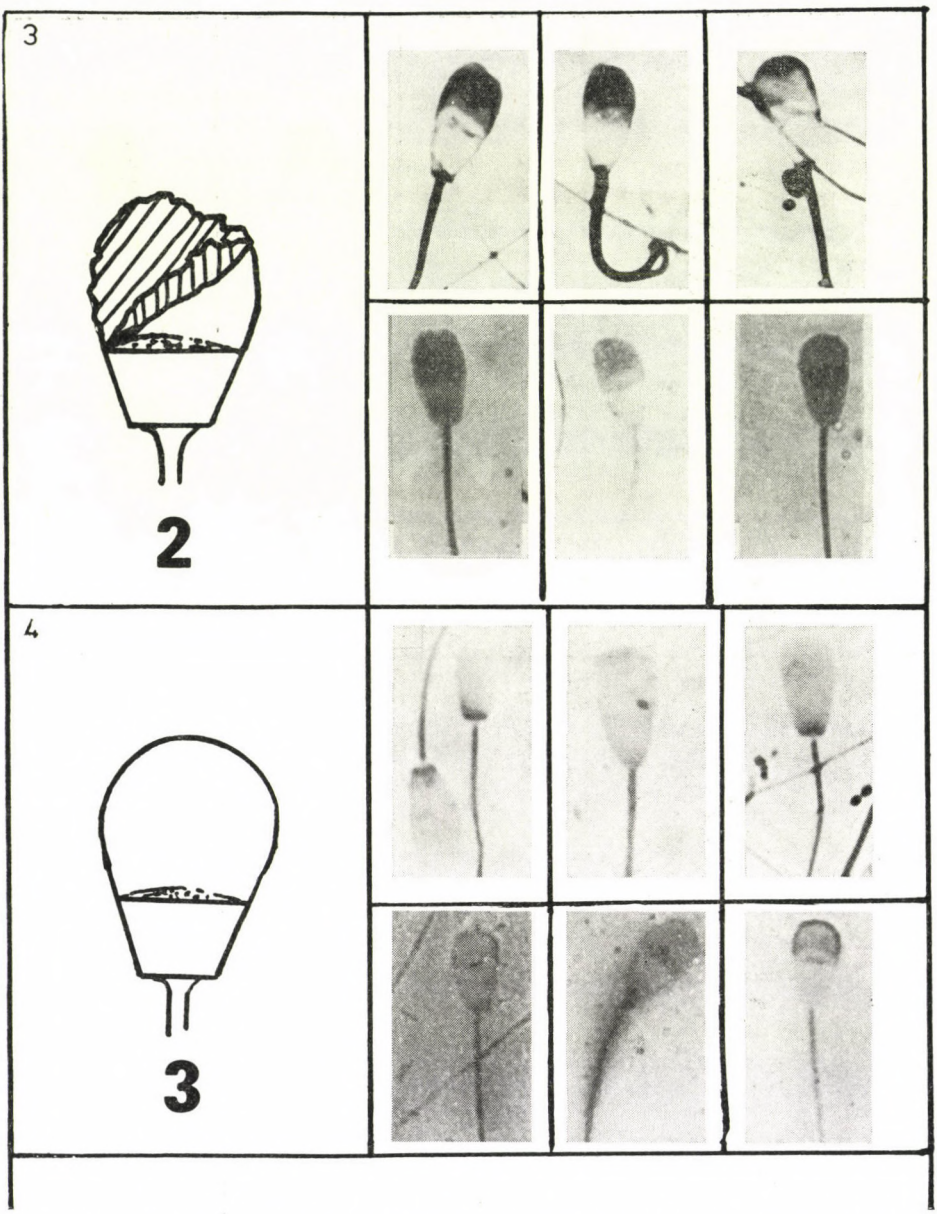

Fig. 3. Line drawing of sperm acrosome picture (score 2, completely damaged acrosome). Light micrographs of A. I. bulls' spermatozoa acrosomes (score 2, completely damaged acrosomes). Phase contrast ×1350

Fig. 4. Line drawing of sperm acrosome picture (score 3, sperm with complete loss of acrosome). Light micrographs of A. I. bulls' spermatozoa acrosomes (score 3, sperm with complete loss of acrosome). Phase contrast ×1350

Results

The percentage distribution of scores calculated for smears of fresh spermatozoa is shown in Table I. In the semen of most bulls, the proportion of normal sperm acrosomes was 91% (undamaged acrosomes, 0). When semen had been equilibrated for 3 1/2 h (stage B), the acrosome score showed 87% normal sperm acrosomes. Frozen-thawed spermatozoa acrosome (stage C) showed a highly significant change in the frequency of normal acrosomes. Spermatozoa stored in the frozen state for 24 h (stage D) were not significantly different in distribution from C-stage spermatozoa, but highly significantly ($P < 0.001$) differed from spermatozoa of stages A and B (Table I).

Table I

Percentage distribution of acrosomes of A. I. bulls' spermatozoa, sampled in stages A–D

Treatment	Acrosome score %			
	0	1	2	3
A	91	6	2	1
B	87	9*	3	1
C	67**	11*	13*	9*
D	71**	10*	7*	12*

* significant ($P < 0.05$).
** highly significant ($P < 0.01$).

Table II

The effect of acrosome score (0–3) of bull spermatozoa on semen characteristics during and after the freezing processes

	Treatment	Number of bulls	Semen quality		
			Motility %	Motility after thawing %	Fertility %
A	Acrosome score (0)	90	72	41	47.4
	Acrosome score (1–3)	10	64*	36*	37.5*
B	Acrosome score (0)	85	65	40	49.6
	Acrosome score (1–3)	15	61.5	37.2*	43.4*
C	Acrosome score (0)	69	62	45	51.6
	Acrosome score (1–3)	31	55*	30**	38.0**
D	Acrosome score (0)	65	65	40	49.3
	Acrosome score (1–3)	35	60*	34.5*	37.2**

* significant ($P < 0.05$).
** highly significant ($P < 0.01$).

The results clearly show that the frequency of defective spermatozoa and of acrosome disruption increased under the freezing conditions. There was a significant decline of sperm motility during the freezing processes, and the significant result obtained regarding the sperm acrosome score is of special interest. The samples of normal acrosome picture were highly significantly more fertile than the samples showing acrosomes of scores 1–3.

From the results shown in Table II a positive correlation was established between the stage of disruption of acrosomes and sperm fertility.

Discussion

It has long been suggested that the morphology of spermatozoa is an important parameter in the routine evaluation of male fertility (Eliasson, 1975). The morphology of bull spermatozoa is well-known. Many different types of spermatozoa have been described in detail but the acrosome changes in different phases of freezing in liquid nitrogen and thawing have not been investigated so far.

Acrosome formation begins very early in spermatogenesis and shows configurational changes that continue in spermatozoa passing through the epididymis. The plasma membrane overlaying the acrosome has been a subject of interest in recent years. Watson and Martin (1972) succeeded in classifying the state of the acrosomal structures reproducibly. In their studies, freezing was accompanied by a progressive derangement of the acrosome. Our present observations have confirmed these results. The decline in staining intensity and in contrast after freezing (Watson, 1975) may indicate loss of stainable material from the membranes or the contents of the acrosome and would, therefore, be further evidence of acrosomal damage. The present results of the decline of sperm motility and motility after thawing, and the highly significant decrease of fertility prove the suggestion of Watson and Martin (1972) and Watson (1975), and the results obtained by us (Moustafa A. R. Ibrahim, 1979, 1980) on the release of some chemical substances and enzymes into the surrounding medium as indication of acrosomal damage. The fertility of frozen spermatozoa is probably influenced by several factors, among them the survival of spermatozoa in the dilutions, during freezing processes and in the reproductive tract. The acrosome scoring used by us is suitable for evaluating the harmful effects of the diluents, glycerolation, semen handling, equilibration time needed for the sperm of different species to survive in diluents, and of temperature treatments during frozen storage.

The present results agree with those of Claus (1976) who showed that the increasing concentrations of dimethyl sulphoxide (DMSO) between 2% and 10% in the extender caused a significant increase in motility and a significant

drop in the proportion of normal acrosomes. Testing NAR (normal apical ridge) in frozen semen does allow a better prediction of the later fertilizing capacity than MOT (motility) does. These facts confirm our finding that acrosome scores (0-3) may be used as a parameter better than motility control for sperm fertility. Carlos (1975) proved the importance of the microscopic examination of acrosome morphology for finding the optimum diluent concentration for the protection of acrosomal structure. We conclude that, by light microscopy, the integrity of the acrosomes can be assessed, and the technique of acrosome scoring is useful for estimating the state of the acrosome structure. The method can be used in routine semen evaluation as a further criterion, independent of motility, of the intactness of the cells. It is also a good criterion for assessing the abnormalities of the acrosome reaction under several conditions.

We suggest that these results and methods can be useful in further studies on sperm maturation, capacitation and fertilization, and for detecting acrosomal changes and defective acrosomes.

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EFFECTS OF DEEP-FREEZING ON BOAR SPERM

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Boar semen was obtained by the hand method from 50 boars, descendants of four sires (Duroc, Estonian, GDR Lowland and German Öves boars). In the breeding period of 1979, 1218 doses of deep-frozen boar sperm were made from 190 ejaculates in special rubber tubes (artificial straws). For deep-freezing, 11% lactose solution was used as diluent, then 0.1% skimmed milk-powder and freeze-protective (cryoprotective) materials, glycerol and Orvus Es Paste were added. The deep-frozen sperm was thawed in water bath of 52 °C for 52 s, while the sperm warmed up to 20 °C, then it was diluted in thawing liquids (Beltsville or OLEP Liquid), and sows were inseminated. Pregnancy was determined by ultrasound 40 days after insemination. Of the 397 sows inseminated with deep-frozen boar semen, 184 (46.3%) farrowed, with an average litter size of nine pigs. The morphology of the boar sperm head was examined before and after deep-freezing, using light and electron microscope. Significant differences were observed with respect to age and sire.

Polge et al. (1970) applied the technology of bovine sperm freezing to the freezing of boar sperm. The thawing results were remarkable (25 to 30%). The main aim of research conducted by other authors (Roy, 1955; Bane, 1959; Baier, 1962; Bader, 1964; Rostel, 1976) was to determine the optimum equilibration time of sperm to freezing. The materials used for conservation were considered to be suitable even for deep-freezing and for durable storage of diluted centrifuged sperm. Plastic tubes or glass ampoules were used for storing deep-frozen boar sperm, but these technologies showed no improvement (Carbo and Einarsson, 1971; Boehnke, 1973). Since 1970, boar sperm has been deep-frozen in granulated (pelleted) form (Einarsson et al., 1971; Graham et al., 1971; Rohloff and Allmelling, 1971; Paquignon et al., 1972; Klenner 1975).

When the method of Pursel et al. (1972) was used, the motility of thawed sperm increased to 35%, and the proportion of intact acrosomes rose to 50 to 55%. Eleven sows which were slaughtered 10 days after insemination showed fertilized ovules.

Westendorf et al. (1977) examined the freezing of sperm in polyamide plastic tubes (Hülsenberg Pailette) and achieved a 38% litter rate. The thawed sperm showed 30 to 35% motility and 55 to 60% normal acrosome rate.

Schrader et al. (1977) compared the method of pelleting (Pursel et al., 1972) with the Westendorf method of artificial straw. By the method of split-sample, half of the ejaculates were granulated and the other half were examined in plastic tubes. Motility proved to be better with artificial straw than with the pellet technology. The rate of intact acrosomes did not show a significant difference. The advantage of this method over the pellet one is that the artificial straws can be signed originally, so their identification is sure. Thawing is difficult if the sperm is granulated, while the sperm frozen in artificial straws is simple to thaw. In spite of the good results, the artificial straw method has not been widely applied because of its high costs.

The present study in Hungary was started on the basis of the experiments conducted in West Germany (Neustadt a. d. Aisch, Hülsenberg) in 1979.

The aims of the examinations were to work out a technology for deep-freezing boar sperm in large artificial straws, and to improve biological parameters and reproducibility. These are very important factors in the practice. Practical use of deep-frozen boar sperm makes possible to use sperm from excellent boars apart from the time. Boars, descendants of which are controlled, can be used in stock-breeding systematically, and special characters, stocking of genes and boar SPF technology can be introduced in the practice.

Materials and methods

The experimental animals were 50 boars, 1.5 to 5 years of age, from four sires (Duroc, Estonian, GDR Lowland and German Öves) used for semen collection from the Artificial Insemination Centres of Budapest and Füzesabony, Hungary. The animals were prepared and semen was obtained by hand from the first phase, on the average 80 to 100 ml. Semen was collected in centrifuge tubes of 20 ml, warmed up to 35 °C and filtered through double gauze. Then the tubes were placed in a water bath at 35 °C for 10 min. During this period, motility and morphology of the sperm, rate of intact acrosomes and number of sperm were registered. Only sperm reaching 75 to 80% motility were frozen. Abnormal sperm and sperm of low motility in 20 to 25% or more were discarded. Morphology of the sperm was examined in preparations stained with 1% methyl violet and dried ($\times 1350$).

The sperm of normal construction and of abnormal form were noted; in the latter, head, neck and tail deformations were also indicated. Abnormalities of sperm were grouped as follows: (1) giant and pigmy forms; (2) deformed heads; (3) abnormal joining to connecting part; (4) doubled formations; (5) other formations on head, neck and tail; (6) wound tail; (7) missing cap, and disorder in acrosomes.

The sperm concentration was determined by a colorimetric method. The average values of the density of ejaculates are 0.1 to 0.5×10^9 per ml. The

semen was extended in a diluent and allowed to equilibrate for 2 h at room temperature before cooling and freezing. The diluent consisted of 120 g glucose, 7.4 g selecton B_2, 7.5 g sodium citrate, 2.4 g sodium hydrogencarbonate, 1.0 g sigmamycin diluted in 2000 ml double-distilled water. Cooling and freezing dilutions were made during this time.

Table I

Composition of the cooling and freezing dilution (quantity needed for ejaculates)

Cooling dilution	Quantity					
Lactose solution (11%)	40	80	100	120	160	200 ml
Egg yolk	10	20	25	30	40	50 ml
Total volume	50	100	125	150	200	250 ml
Enough for	1 to 2		2 to 3		3 to 4	5 ejaculates

Freezing dilution	Quantity				
Cooling dilution	46	92	138	184	230 ml
Orvus Es Paste	1	2	3	4	5 ml
Glycerol	3	6	9	12	15 ml
	50	100	150	200	250 ml
Enough for	1 to 2		3 to 4		4 to 5 ejaculates

The cooling dilution (Table I) was pre-cooled to 15 °C and the freezing dilution to 4 °C. The sperm was placed for 2 h in a thermostat at 15 °C for equilibration, then it was centrifuged at 540 *g* for 10 min. In this step, seminal plasma separated from sperm and gathered as a pellucid liquid above the sedimented cells. Seminal plasma ranged from 185 to 190 ml, and sperm from 10 to 15 ml, in volume.

The centrifuged sperm cell mass was diluted to the final volumes indicated in Table II. Semen was cooled to 5 °C during 1.5 h and freezing diluent was added to yield the concentration of 1×10^9 spermatozoa per ml.

The sperm was diluted with freezing dilution (Table II), precooled in artificial (PVC) straws and equilibrated for 20 min. The straws were 30 cm in length, 9 mm in diameter, 0.6 mm in thickness, final volume of 10 ml, closed by 2 steel balls from the two ends. The data of the boar and information were indicated on the straw.

The straws were transferred to containers of liquid nitrogen on ramps being over the liquid level of 5 cm and frozen for 20 min in nitrogen steam of —120 °C. After freezing, the artificial straws were placed in containers of —196 °C liquid nitrogen for storage. After 24 h, the motility of the deep-frozen sperm, the rate of normal acrosomes and the progressive motility were determined. Before insemination the deep-frozen semen straws were thawed in water bath of 52 °C for 52 s. During this time, the temperature of both the

Table II

Dilution of sperm samples depending on the total number of spermatozoa in prediluted settling

No. of spermatozoa in prediluted settling $\times 10^9$	Volume of cooled sperm ml	Volume of freezing dilution ml	Final volume of frozen sperm ml	Obtainable No. of artificial straws
5.0 to 9.5	4.0	2.0	6.0	1
10.0 to 14.5	8.0	4.0	12.0	2
15.0 to 19.5	12.0	6.0	18.0	3
20.0 to 24.5	16.0	8.0	24.0	4
25.0 to 29.5	20.0	10.0	30.0	5
30.0 to 34.5	24.0	12.0	36.0	6
35.0 to 39.5	28.0	14.0	42.0	7
40.0 to 44.5	32.0	16.0	48.0	8
45.0 to 49.5	36.0	18.0	54.0	9
50.0 to 54.5	40.0	20.0	60.0	10
and so on				

Table III

Heat resistance of boar sperm deep-frozen in two types of diluents

Boar	Deep-frozen sperm at 0 h				Deep-frozen sperm after 5 h at +37 °C			
	Motility		Normal acrosome		Motility		Normal acrosome	
	a	b	a	b	a	b	a	b
A 023	35.4	33.1	65.3	61.4	25.0	23.3	23.3	31.6
A 057	40.2	37.3	64.2	61.2	20.0	21.6	23.6	28.3
A 074	36.2	34.5	62.5	65.0	21.6	24.6	29.3	39.2
A 110	35.5	31.4	65.0	67.1	19.2	21.3	27.2	31.3
A 111	37.2	34.4	58.0	60.4	21.6	26.2	29.3	36.5
A 112	37.5	35.5	60.5	66.5	20.6	22.6	26.5	31.6

a = lactose diluent; b = milk-powder diluent

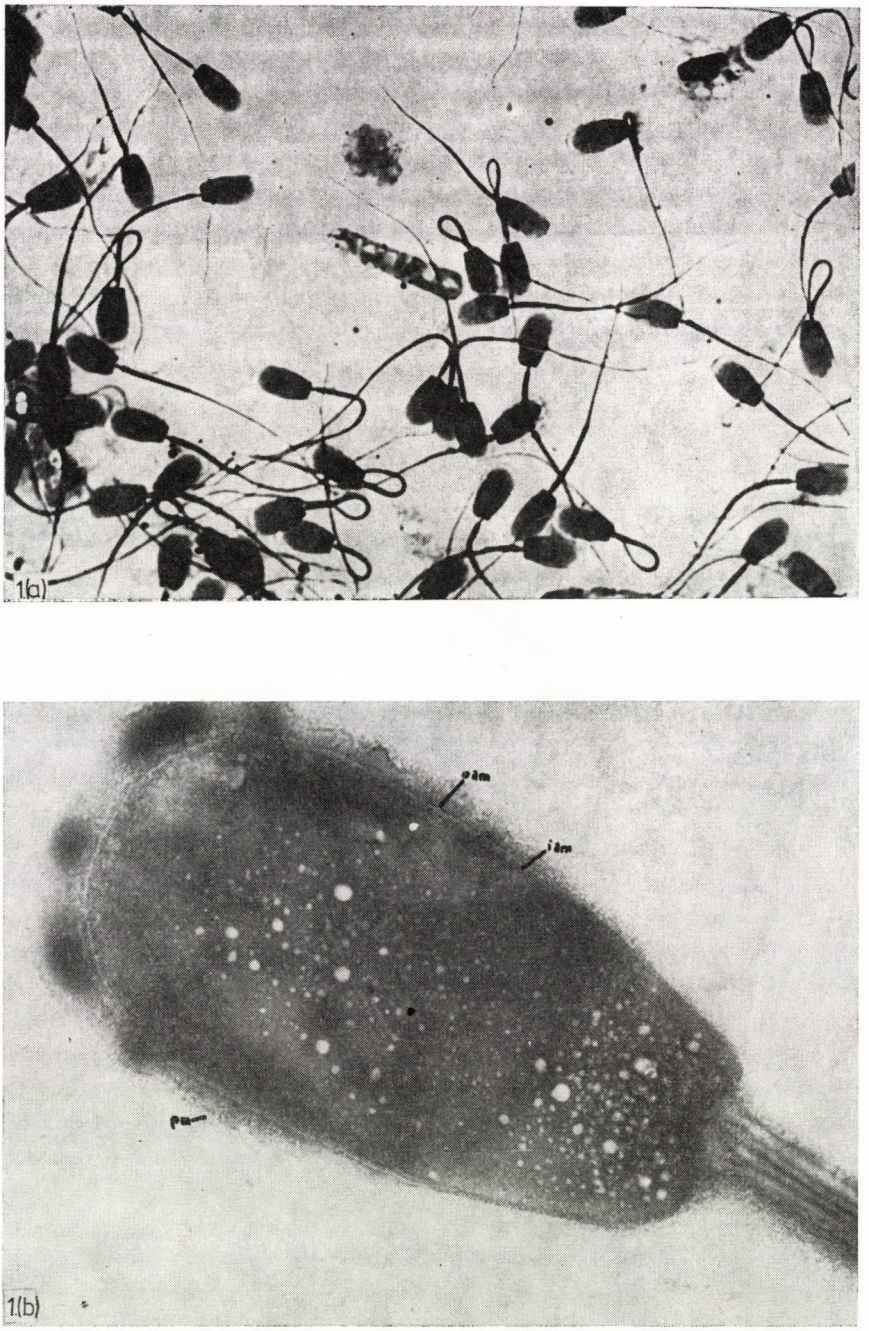
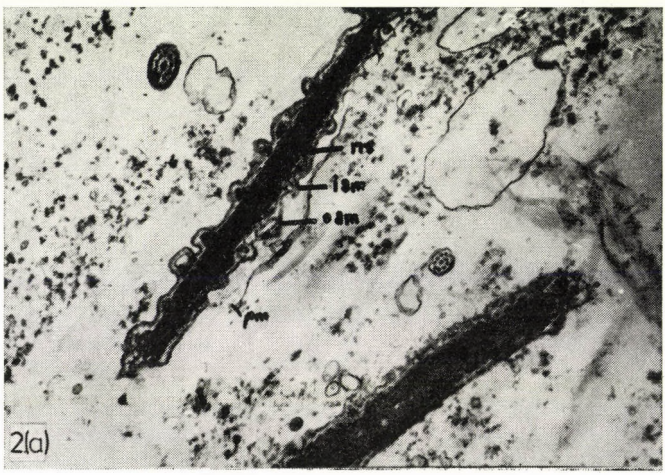
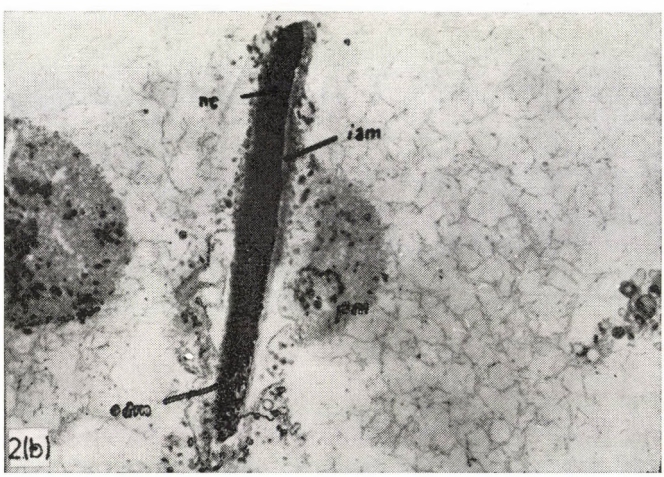


Fig. 1. a Stained boar spermatozoa showing normal and damaged sperm acrosomes — winding and breaking of the tail, abnormal sperm head. ×1350. *b* Electron micrograph of native normal boar sperm head and acrosome, outer acrosomal membrane *(oam)*, inner acrosomal membrane *(iam)* and plasma membrane *(pm)*. ×37,000

sperm and the thawing dilution rose to 20 °C. The layer of the sediment was cooled to 20 °C and stored at this temperature. It was used as a dilution which contained 50% seminal plasma. Before being used, it was thawed, and centrifuged for 20 s at 6000 *g*. The upper sediment, free from sperm, was diluted to contain 30% seminal plasma. Seminal plasma treated in this way can be stored at 4 °C for 4 days. Semen was thawed in either Beltsville thawing solution (Pursel et al., 1972) or OLEP Diluent (Larsson and Einarsson, 1976).

The diluent was added (70 ml) to thawing spermatozoa, and the sows were inseminated twice at a 12-h interval. Pregnancy was determined by ultrasound 40 days after insemination. An additional diluent containing 11% powdered milk, 20 ml egg yolk and 80 ml distilled water was used for cooling





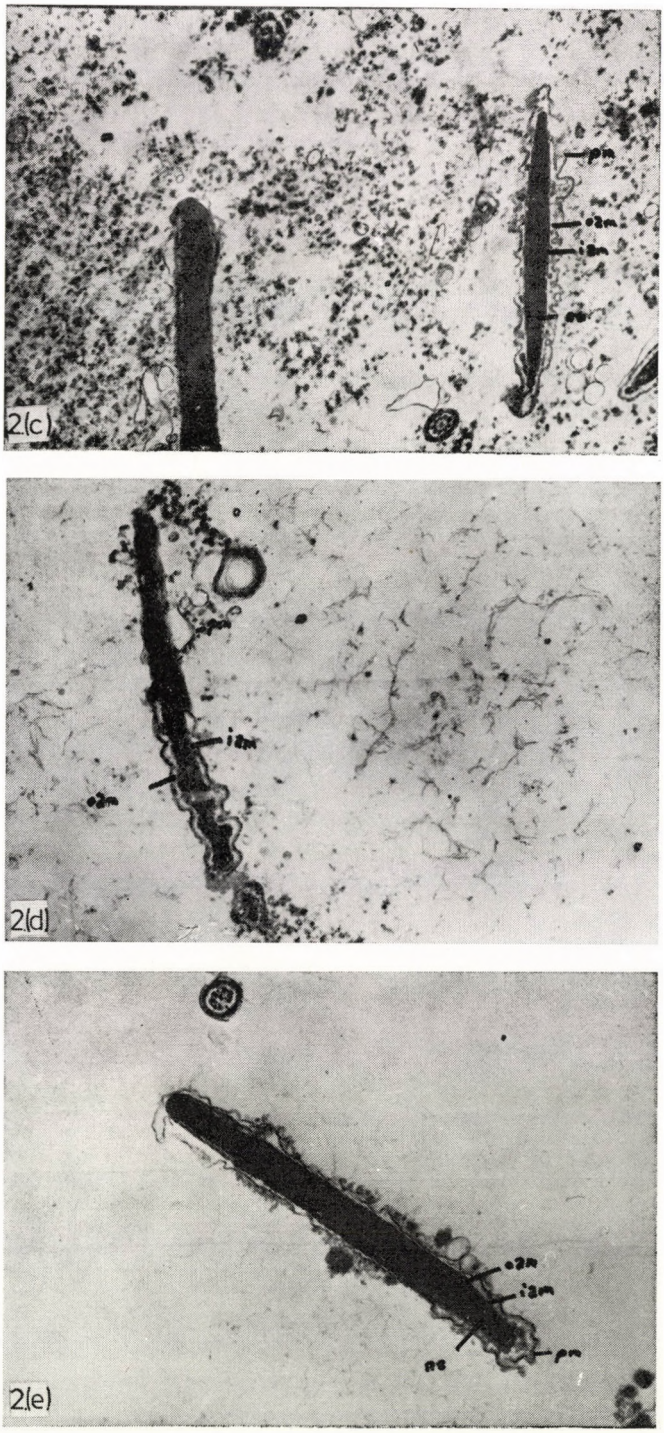


Fig. 2. a–e Electron micrographs of portions of boar sperm. Note outer *(oam)* and inner acrosomal membranes *(iam)* and nuclear envelope. The *oam* can be identified by the stained acrosome contents. The plasma membrane is widely separated from the *oam* (Fig. 2*b–d)*, note the damaged acrosomal membrane. ×37,000

and freezing ejaculates of 6 boars for comparison with the lactose diluent. Half of these ejaculates was treated with lactose and the other half with milk-powder dilution. The ejaculates were then frozen. Heat resistance was determined in the sperm deep-frozen in the two kinds of dilutions at 37 °C for 5 h as follows: (1) 0.6 ml of each thawed sperm sample was poured into a 10-ml tube containing 8 ml of thawing dilution of the same temperature. (2) The samples were placed in a water bath at 37 °C, and (3) motility and number of intact acrosomes were determined (Table III).

Results

Fresh semen samples from the 50 boars showed on the average 75 to 80% motility and the rate of normal acrosomes was 92 to 95%. After deep-freezing and thawing spermatozoa, good quality semen was maintained in only 25 of the boars.

The first morphologically demonstrable damage occurred during centrifugation of the diluted semen. The damage was characterized by wound tails and disappearance of agglutination and motility. Winding and breaking of the tail appeared at different levels and were used for evaluating the sperm quality. After sperm agglutination, i.e. head-head, head-tail and tail-tail agglutination, had occurred, the numbers of moving spermatozoa and the frequencies of the following phenomena were reduced significantly: abnormal acrosomes in native sperm, abnormal-headed and abnormal-tailed spermatozoa in native sperm, normal acrosomes in diluted sperm at 5 °C, motility in thawed sperm, and normal acrosomes in thawed sperm. The effects and damages are shown in Figs 1 to 3. The sperm samples from 25 boars that were

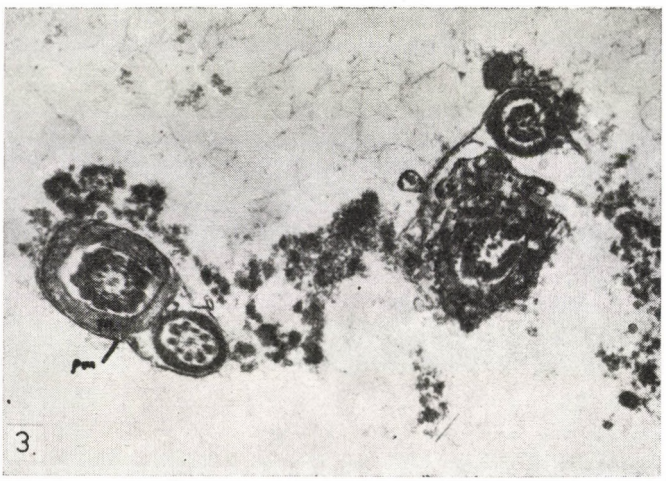


Fig. 3. Lead-stained section through portions of normal boar sperm tail; only the plasma membrane *(pm)* is heavily stained; mitochondrial *(m)* membranes are not stained. ×37,000

not suitable for deep-freezing showed further damage during freezing at —120 °C. Sperm motility from these animals was 15 to 20%, and the proportion of sperm having intact acrosomes was very low, 15 to 30%. At first the outer membrane of the acrosome was damaged, but later fusion of outer and inner membranes, deformation of heads and formation of plasma drops also occurred. Semen examination was repeated three times and yielded similar results. The 25 boars were examined further. Table IV gives information on the biological characteristics of ejaculates from the 25 boars presenting good results, and on the changes during deep-freezing.

Data in Table IV prove that the average number of spermatozoa obtained from first-phase ejaculation was 37.4×10^9. There were significant differences ($P < 0.05$) between sires and ages. The number of spermatozoa averaged 45×10^9 for the Duroc boars, while for Estonian and GDR Lowland boars it was about 34×10^9. Total sperm number of German Öves boars was 36×10^9. Differences by age were also considerable: ejaculates of young boars contained 32 to 35×10^9 spermatozoa, while those of old boars had 35 to 48×10^9 spermatozoa.

During our examination, 1218 doses of boar sperm were deep-frozen in artificial straws. Nine hundred thawed artificial straws showed 37% motility; the proportion of intact acrosomes was 60.4%; of the 397 sows inseminated during one breeding cycle, 184 sows (46.3%) farrowed. There were no differences in results between sperm thawed after 30 days and that thawed after 12 months, or in the litter size obtained.

Discussion

One of the important factors in the deep-freezing of boar sperm is the maintenance of motility. On motility examination, Westendorf et al. (1977) proved that the head is 8 μm in length and 4 μm in width, the neck is 12 μm in length and the tail is 30 μm in length. The quick reduction of the boar sperm motility can be explained by the fact that the ratio of head to tail is less favourable than in bovines, viz. the head of the same size is associated with a tail 40% shorter than that found in bovines. Boar spermatozoa move around in a circle of 100 μm, and the duration of this movement depended on the time of storage: the longer the storage, the shorter was the duration of the movement. Larsson and Einarsson (1976) found that motility of thawed sperm can be increased by adding 10 mmol caffeine. Gustafsson and Einarsson (personal communication) showed that prostaglandin F_2 added to the thawing solution accelerated the movement of sperm.

Another important factor is acrosome morphology, which plays a key role in fertilization biology (Hillmann, 1972; Jones, 1973; Müller and Brandl,

Table IV

Biological and physical traits used for

No. of boar	1	2	3	4	5	6	7	8	9
A 023	4	85	36.2	75	7.2	2.5	4.7	0.2	1.0
A 057	3	80	32.2	75	7.3	4.3	3.0	0.3	1.1
59	14	100	36.2	80	6.5	2.7	3.3	0.2	0.3
A 074	4	90	35.2	75	6.5	2.5	4.2	1.3	0.0
A 077	3	80	34.2	78	9.6	2.3	7.3	0.0	0.0
A 102	3	90	32.2	76	6.0	2.6	6.5	6.0	0.0
A 103	15	100	36.1	80	7.8	2.9	5.0	0.5	0.5
A 108	4	90	38.2	75	7.2	2.6	4.4	0.2	0.5
A 110	14	90	42.2	80	7.6	3.1	3.6	0.2	0.1
A 111	14	96	48.3	80	7.2	2.9	3.5	1.0	0.5
A 112	14	95	42.2	85	7.5	3.0	3.9	0.3	0.2
A 113	14	90	36.2	80	9.0	3.2	3.0	0.4	0.4
A 139	5	90	36.5	76	8.5	3.0	5.5	1.5	1.5
A 141	5	90	30.2	75	9.6	3.6	6.0	2.0	1.0
A 143	5	95	36.8	76	9.1	3.2	5.6	1.6	1.4
A 144	5	90	37.5	80	9.2	3.1	5.4	1.5	1.2
A 149	10	95	38.3	80	7.6	3.0	4.1	1.5	0.5
A 150	8	95	36.5	80	6.1	2.7	3.6	1.0	0.3
A 151	4	80	32.5	75	7.0	2.4	4.5	0.3	0.6
A 158	6	90	38.2	80	7.2	2.8	4.0	1.5	0.4
A 159	6	90	38.8	80	7.5	2.6	3.8	1.6	0.3
A 195	10	90	44.2	80	7.4	3.0	3.7	1.0	0.5
A 196	10	95	43.5	80	7.6	2.8	3.5	1.0	0.6
A 321	4	80	36.6	76	7.0	2.0	4.3	0.2	0.6
1069	6	90	36.5	76	6.8	2.7	3.5	1.0	1.0
I	7.6	90.2	37.4	78	7.6	2.8	4.3	1.0	0.5
II	7.3	91.1	35.3	73	9.0	3.0	5.0	3.2	2.8

I: Average values for semen of good boars; II: Average values for semen of "bad" boars; 1: No. of ejaculates; 2: Volume of ejaculate (ml); 3: Total number of spermatozoa in the ejaculate, $\times 10^9$; 4: Moving spermatozoa, per cent; 5: Plasma drops hanging on spermatozoa, per cent; 6: Spermatozoa with plasma drop of proximal position in native sperm, per cent; 7: Spermatozoa with plasma drop of distal position in native sperm, per cent; 8: Spermatozoa with abnormal acrosome in native sperm, per cent; 9: Abnormal-headed spermatozoa in native sperm, per cent;

1975). The acrosome (cap) formula covered by double membrane covers 3/4 of the head (see Fig. 1b). During deep-freezing, it is mainly the acrosome which is damaged (Fig. 2a, b, c, d and e). Damage in construction may occur on its

evaluating deep-frozen boar sperm

10	11	12	13	14	15	16	17	18
6.7	94.2	35.0	65.0	1.2	5.9	2.4	4.7	6.0
6.0	87.5	40.0	64.2	1.3	5.9	1.7	3.5	5.3
6.0	92.5	40.0	60.0	1.1	5.9	2.5	3.8	6.0
7.1	93.3	36.2	62.5	1.2	5.9	3.3	5.0	5.8
5.0	82.5	38.0	60.0	1.1	5.2	2.5	4.0	5.6
0.0	90.0	36.0	58.0	0.8	5.2	1.9	2.9	5.3
5.0	92.5	36.5	60.0	1.1	5.9	2.0	4.0	6.0
6.7	91.0	36.2	61.0	0.9	6.1	2.0	4.5	6.3
5.8	86.5	35.0	65.0	1.1	6.5	2.5	3.0	7.0
5.9	90.5	37.0	58.0	1.2	6.5	2.7	4.0	8.0
6.0	91.0	38.0	60.0	1.1	6.0	2.4	4.1	7.0
5.8	92.0	38.0	61.5	1.2	5.9	2.5	3.8	6.0
4.6	91.5	36.0	56.0	1.3	5.9	1.8	3.6	6.0
4.5	92.0	35.5	56.0	1.2	6.0	1.7	3.5	5.0
4.6	86.0	36.0	60.0	1.1	6.0	2.0	3.4	6.1
4.4	88.5	36.0	58.0	1.3	6.0	1.8	3.7	6.2
6.0	91.5	40.0	61.0	1.2	6.1	2.1	3.6	6.3
5.6	90.5	36.5	60.5	1.3	6.0	1.5	3.5	6.0
6.0	92.5	38.0	58.5	1.3	5.9	1.6	5.2	5.4
5.6	91.0	38.0	60.5	1.2	6.1	1.8	3.7	6.4
5.8	92.5	35.5	62.0	1.3	6.1	2.0	4.0	6.5
6.0	90.5	36.8	61.0	1.2	6.0	2.0	3.4	7.3
5.4	91.5	38.5	60.5	1.1	6.1	2.5	4.0	7.2
6.1	91.5	36.0	61.0	1.3	5.9	2.0	4.3	6.0
5.5	91.6	35.5	60.0	1.4	5.9	1.8	3.6	6.0
5.4	90.6	36.9	60.4	1.1	5.9	2.1	3.8	6.2
7.2	70.8	22.9	41.4	0.9	5.6	1.1	2.9	5.1

10: Abnormal-tailed spermatozoa in native sperm, per cent; 11: Normal acrosomes in diluted sperm at 5 °C, per cent; 12: Motility in thawed sperm, per cent; 13: Normal acrosomes in thawed sperm, per cent; 14: Density of semen in thawed sperm, million/mm^3; 15: Number of spermatozoa in one artificial straw ($\times 10^9$); 16: Number of moving spermatozoa in one artificial straw ($\times 10^9$); 17: Number of spermatozoa with normal acrosomes in one artificial straw ($\times 10^9$); 18: Number of artificial straws

apical part, the outer acrosome membrane can be deformed, it mixes with the inner membrane or parts from the head. This effect is due to the release of enzymes ensuring penetration. Low fertility rate of the boars corresponds

to increased rate of disruption of the acrosomal membrane, visible by light microscope. Acrosome damage similar to that found by Pursel et al. (1972) was also found in motile sperm of boar. Further improvement in deep-freezing of boar sperm can be expected only by increasing the percentage of spermatozoa surviving thawing, and preventing litter size from being reduced for some unknown physiological reason.

To solve this problem, a method is to be worked out according to which the motility is 45 to 50% after thawing. Better results can be expected from artificial seminal plasma prepared from OLEP one. Presently, there is a significant improvement in fertilization of sows by flowing sperm. According to the present storage technology, the sperm is used up within 24 to 48 h. So, 50% of the delivered sperm is used for fertilization. If deep-frozen sperm is used for fertilization, the conception rate is 80%, after first or repeated inseminations. Our aims for the future are to use machines in handling artificial straws, to increase the percentage of motile sperm after thawing and to improve litter size by increasing the dose of sperm in the ejaculate.

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COMPARATIVE BIOCHEMICAL AND PATHOLOGICAL STUDIES ON ACUTE RUMINAL ACIDOSIS INDUCED BY MOLASSES AND GRAIN FEEDING IN BUFFALO CALVES *(BUBALUS BUBALIS)*

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Buffalo calves were fed with a single dose of molasses orally (Group 1) or with crushed wheat grains intraruminally (Group 2). In Group 1 there was an initial increase in the lactic acid concentration of ruminal fluid and blood while in Group 2 there was a continuous increase in lactic acid content throughout, culminating at a 2-fold–3-fold increase. Molasses feeding caused hypoglycaemia while the grain-fed animals developed hyperglycaemia. Concurrent to these changes in biochemical parameters, there were differences in quality as well as magnitude of histopathological changes in the cerebrum, exocrine and endocrine pancreas, rumen, reticulum, omasum and liver. The histopathological changes were correlated with the biochemical changes.

Rumen acidosis due to sudden access to carbohydrate-rich diets occurs commonly all over the world. Different workers have studied the problem from different angles, but there are only few publications on histomorphological changes in the organs of the affected animals. Dunlop and Hammond (1965), Ahrens (1967) and Dirksen (1970) reported histopathological changes in acidotic cattle, Delak and Adamić (1959), Hartig and Hebold (1973), Vestweber and Leipold (1974) and Dshurov (1976) recorded the same in sheep. In the buffalo *(Bubalus bubalis)*, such changes have only been recorded by Nauriyal et al. (1978). The present work constitutes part of a comprehensive study dealing with biochemical and histomorphological changes in different tissues of acidotic buffaloes. In this study, the degree of involvement of the organs as well as the histomorphological changes were comparatively examined in buffaloes with ruminal acidosis induced by feeding of crushed wheat grains and that induced by feeding of molasses.

Materials and methods

Ruminal acidosis was induced in four buffalo calves (Group 1) aged 2-2 1/2 years by oral feeding of molasses, mixed with water, at the rate of 10 g/kg body weight. In five animals of the same age range (Group 2), acidosis was induced by intraruminal feeding of crushed wheat grains at the rate of

50 g/kg body weight. Before feeding, the normal pH of the ruminal fluid and urine, lactic acid content of the ruminal fluid and blood, and glucose concentration of blood were recorded. The pH values were recorded in a systronic GRIPH pH meter immediately after the samples had been withdrawn. Rumen lactic acid and blood lactic acid were estimated colorimetrically by the method of Barker (1961). Blood glucose was estimated using Haden's modification of the Folin-Wu method as described by Frankel et al. (1970). The same parameters were assessed at 24-h intervals on the next five days. Of the four animals of Group 1 and the five of Group 2 one (72 h) and two animals (96 h and 120 h), respectively, died. One animal of Group 1 was killed 72 h post feeding. The parameters of these animals are shown in Table I. The other animals survived the five-day period and later on responded to treatment

Tissues were collected in 10% buffered neutral formalin, Zenker formalin and alcoholic formalin solutions immediately after death of the four animals. The tissues were processed routinely and paraffin sections 5 μm in thickness were cut. The following staining techniques were employed: haematoxylin and eosin (Luna, 1968); PAS — alcian blue (pH 1.0, Luna, 1968), Best's carmine for glycogen (Luna, 1968) and diamine silver for enterochromaffin cells (Lillie, 1965).

Results

The oral feeding of molasses induced acidosis in the rumen more quickly than intraruminal feeding of crushed wheat grains. In the molasses-fed animals there was a sudden drop in the pH of ruminal fluid, which, however, improved gradually, whereas in the grain-fed animals there was a gradual increase in acidosis throughout the entire period. A similar trend was observed in the lactic acid content of ruminal fluid and blood, and also in the blood glucose concentration (Table I).

Table I

Biochemical changes observed in acidotic buffaloes fed with molasses (Group 1) and grains (Group 2)

Time of sampling h	Rumen fluid pH		Urine pH		Lactic acid, mmol/l				Blood glucose, mmol/l	
					Rumen fluid		Blood			
	Group 1	Group 2	Group 1	Group 2	Group 1	Group 2	Group 1	Group 2	Group 1	Group 2
0	6.80	6.83	8.05	7.95	0.12	0.12	2.02	1.98	3.18	3.32
24	4.76	5.21	6.55	6.43	9.39	7.94	6.83	5.47	4.18	4.66
48	4.84	4.57	5.60	5.85	9.10	10.75	7.11	7.65	3.80	4.92
72	5.00	4.19	5.73	5.70	8.39	13.50	6.43	9.49	2.64	5.56
96	5.35	4.03	5.95	5.40	7.93	14.80	5.58	11.33	2.52	6.35
120	5.68	3.93	6.60	5.48	6.80	16.14	4.64	12.68	2.92	6.94

In the rumen of molasses-fed animals the cells of certain layers of the stratified epithelium exhibited chromatolysis and nucleolysis. The nuclei of the majority of the cells appeared as large empty vacuoles. The cytoplasm was comparatively dense. There was severe congestion of the blood vessels in all layers of the organ. The tunica intima of capillaries and arteriolae was hyperplastic. Certain parts of the lamina propria within the papillae exhibited alcian blue-positive fibres and ground substance, indicating an increase in acid mucopolysaccharides. Ulceration, exfoliation and necrosis of the surface epithelium so prominent in the grain-fed animals (Fig. 1) were not observed in these.

In the reticulum and omasum of molasses-fed animals almost identical changes were observed. The lamina propria was oedematous and rich in acid mucopolysaccharides. In the secondary papillae, the concentration of the

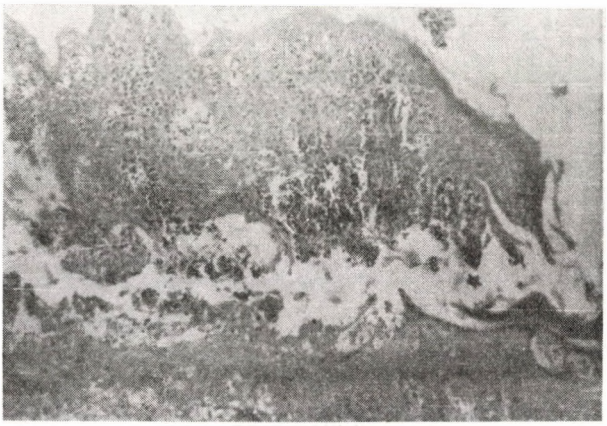

Fig. 1. Rumen showing exfoliation and ulceration in grain-fed animals. H. and E. ×75

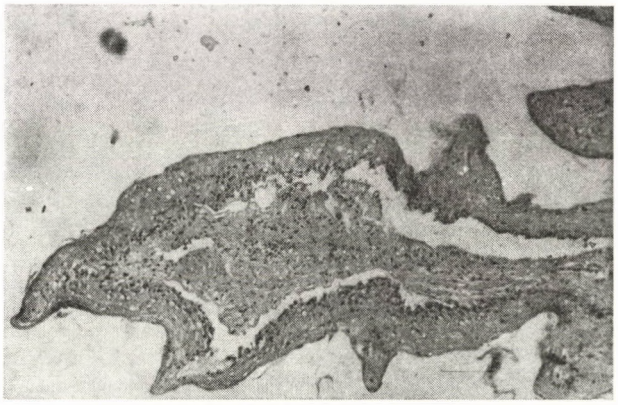

Fig. 2. Reticular papilla showing oedematous separation of the epithelium and vacuolation of epithelial cells in molasses-fed animals. H. and E. ×75

alcian blue-positive material was elevated. The deeper cell layers of the epithelium exhibited cytoplasmic vacuolation (Fig. 2). The epithelium appeared separated from the basement membrane, with a clear oedematous space in between. There was general congestion in all the layers with hyperplastic changes of the tunica intima in arterioles. Perivascular oedema was also conspicuous. In addition, ulceration of surface epithelium was evident in grain-fed animals.

The abomasum, small intestine and large intestine were congested and oedematous. The goblet cells in the intestine were more numerous. The enterochromaffin cells appeared more numerous and were packed with secretion granules (Fig. 3).

The liver in molasses-fed animals exhibited characteristic changes. There was general congestion in all vessels, and oedema. Haemorrhage very pro-

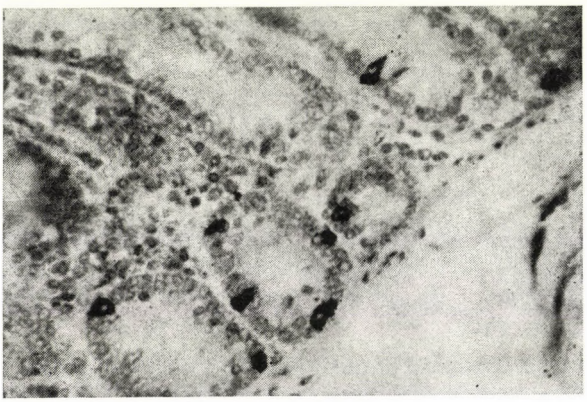

Fig. 3. Duodenum showing enterochromaffin cells laden with secretion granules in molasses-fed animals. Diamine Silver ×400

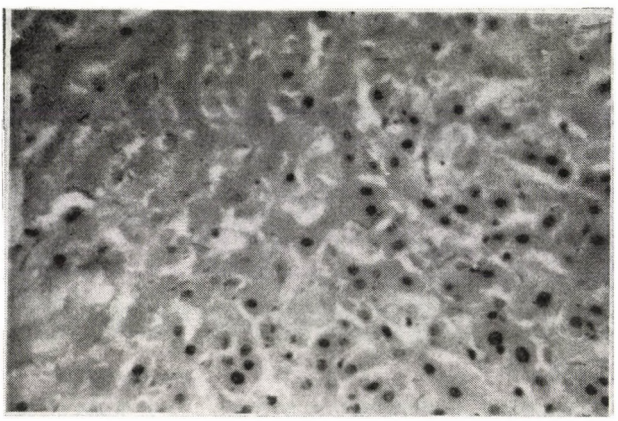

Fig. 4. Liver showing coagulative necrosis of hepatic lobules and pycnotic changes in molasses-fed animals. H. and E. ×400

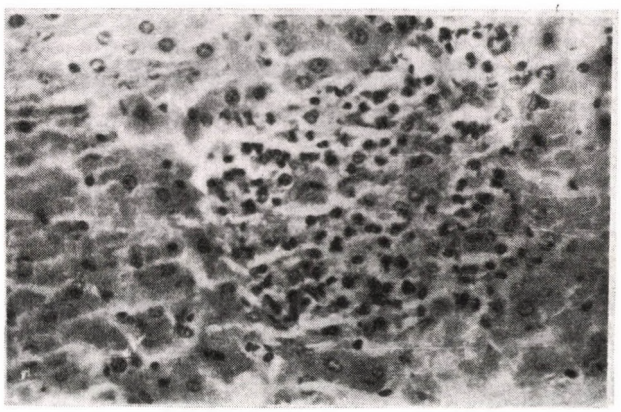

Fig. 5. Liver showing microabscess formation in grain-fed animals. H. and E. ×400

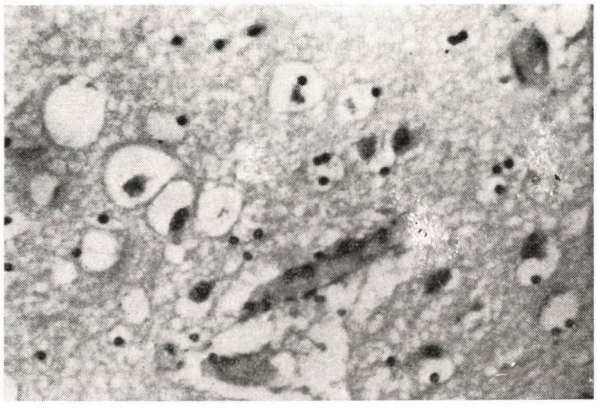

Fig. 6. Cerebral cortex showing perineuronal and perivascular oedema in molasses-fed animals. H. and E. ×400

nounced in grain-fed animals was evident in some parts of the organ. In some of the hepatic cells, the nucleus was pycnotic (Fig. 4). Many hypertrophic cells with enlarged nuclei were present in the organs of molasses-fed animals. In general, the cytoplasm contained little glycogen and appeared foamy. In many lobules the cell cords, sometimes even entire lobules had undergone coagulative necrosis. Microabscess formation and extensive degeneration of the parenchymatous lobules occurred only in grain-fed animals (Fig. 5).

In the lungs, there was severe congestion with oedema, and haemorrhage into the alveolar wall, in both groups of animals. The septal cells were hypertrophic and the alveolar mass was rich in lymphocytes. Emphysema, which was predominant in grain-fed animals, was not observed in the other group.

The cerebral cortex manifested severe lesions only in the molasses-fed animals. There was marked congestion and perineuronal and perivascular

oedema (Fig. 6). There was tigrolysis of cells in many parts of the cortex, along with satellitosis.

The spleen was congested and the subcapsular sinus was narrow. The germinal centres of splenic corpuscles were conspicuous. The pancreas showed general congestion, hypertrophy of the parenchymal cells with enlargement of their basal zones, and degranulation. There was hyperplasia of the islet cells. The epithelial lining of interlobular ducts was denuded at places. In the grain-fed animals, the islet cells were hypertrophic with vacuolated cytoplasm. Hyperplasia was not evident in these cells. The exocrine pancreatic cells were atrophic and, due to congestion and oedema, appeared to have lost their typical acinar arrangement.

The kidneys showed general congestion, thickening of the basement membrane around the glomerular capillaries and hypertrophy of the macula densa cells. Degenerative changes in the tubular epithelium, evident in grain-fed animals, were not present in the other group. In the adrenal gland, histomorphological changes were evident in the zona fasciculata of the cortex, and in the medullary cells. The zona fasciculata cells of grain-fed animals were hypertrophic with a large number of cytoplasmic vacuoles; many cells were binucleated, and polyhedral in outline. The medullary cells, particularly the epinephrine cells of the outer medullary zone, were strikingly enlarged. These changes were mild in the molasses-fed animals. Other organs showed general hyperaemia without any significant cytological change.

Discussion

The biochemical analysis presented in Table I clearly indicates the differences between the two groups of animals. While the grain-fed animals developed severe acidosis due to a steady increase in the lactic acid concentration of both the ruminal fluid and blood as a result of slow but continuous fermentation by amylolytic bacteria, the animals of the other group soon showed an improvement. A marked decrease in the rumen pH to 4.76 (minimum) after 24 h of induction may be due to complete and faster fermentation of simple carbohydrates by rumen microbes, thereby leading to an abrupt increase in rumen lactic acid content and subsequently leading to an increase in blood lactic acid content as a result of lactic acid absorption through the rumen wall (Dunlop, 1972). The blood levels were maintained subsequently by constant absorption from the rumen and inability of liver to convert lactic acid into glucose and also due to decreased tissue oxidation of lactic acid. This is in accordance with the observations recorded in lactic acidosis induced in sheep with simple sugars (Juhász and Szegedi, 1968) and molasses (Losada et al., 1971).

In the grain-fed group, blood glucose concentration increased steadily, leading to hyperglycaemia while in the other group the initial hyperglycaemia was followed by hypoglycaemia, which is a more serious condition. This is similar to the observations of Dirksen (1970); Losada et al. (1971) and Vestweber and Leipold (1974). The hyperglycaemia in wheat-induced acidosis might be due to an increase in glycogenolysis or glyconeogenesis or to reduced utilization of glucose by peripheral tissues (Dirksen, 1970); Kaneko and Cornelius, 1970), or it might be a consequence of decreased level of circulating immuno-reactive insulin. The hypoglycaemia observed in molasses-induced ruminal acidosis might have been caused by a deficiency in volatile fatty acids, particularly in propionic acid, which is the main glucose precursor (Losada et al., 1971), or it might be due to a decreased glyconeogenesis and an increased tissue oxidation of the remaining blood glucose in the acidotic animals.

In general, the histopathological changes were similar to those reported by Dunlop and Hammond (1965), Hartig and Hebold (1973), Vestweber and Leipold (1974) and Nauriyal et al. (1978). Qualitative differentiation of the lesions produced by feeding of grains and molasses has not been reported in any of the works, apart from the fact that Nauriyal et al. (1978) reported more severe pathological lesions in acidotic animals after molasses feeding. The present finding that acidosis appears earlier after feeding of molasses is in agreement with the observations of Nauriyal et al. (1978).

However, in the present study different organs were affected to variable degrees in the two groups of animals. The ruminal mucosa was severely affected in grain-fed animals with exfoliative changes leading to ulceration in many parts of the organ. The same was observed by Nauriyal et al. (1978) in buffaloes, by Dshurov (1976) in sheep and by Ahrens (1967) and Dirksen (1970) in cattle. But in the animals fed with molasses exfoliation and ulceration of the mucosa was not evident in any compartment of the forestomach.

The observations of Ahrens (1967), suggesting that submucosal congestion may be caused by an increased osmolarity of the acidotic rumen contents, are supported by the present results, viz. hyperaemia in general form was observed in the stomach as well as other organs of the body in both forms of acidosis. Toxaemic factors might also be responsible for this, which will also account for the hyperplastic changes of small arterioles.

In the animals fed with molasses, the severity of lesions was of a lesser degree in the liver and pancreas than in animals of the other group. The biochemical changes are consistent with these histopathological findings. Dirksen (1970) reported congestion and necrosis of the liver as an early sign of liver damage in acidotic cattle, and it has also been suggested that invasion of certain bacterial groups through the damaged ruminal mucosa may contribute to this. The rumenitis-liver abscess complex as observed in wheat-induced acidosis might be due to these bacteria as suggested by Jensen et al. (1954).

In the cerebrum, the lesions of molasses-fed animals are identical to those observed by Losada and Preston (1973) and Vestweber and Leipold (1974). The severity of lesions in this organ may be attributed to hypoglycaemia as was suggested by Losada and Preston (1973) and/or to an increased level of histamine (Ahrens, 1967). As was observed by the former authors, the molasses-induced acidosis also revealed a fall in blood glucose after an initial increase. This may also be a contributing factor for the hypertrophic condition of some of the hepatic cells.

The enterochromaffin cells of the gastrointestinal tract secrete several hormones for normal functioning of the stomach and intestines. The stasis observed in these cells may be the reason for atonicity of the reticulo-rumen and small intestine.

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HAEMONCHUS CONTORTUS: THE IN VITRO EFFECTS OF ANTHELMINTICS ON TOTAL GLUCOSE AND GLYCOGEN CONTENTS, AND TOTAL VOLATILE FATTY ACIDS

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Effects of dl-tetramisole and rafoxanide at 50 μg/ml were studied on adult *Haemonchus contortus* in vitro after 8 and 12 h of incubation, respectively. Upon in vitro incubation in drug-free Tyrode solution, glucose levels increased and glycogen content and the amount of total volatile fatty acids (TVFA) decreased. The decrease in glycogen levels might be due to initial expulsion of eggs or to higher rate of glycogen degradation than that of glucose utilization.

Tetramisole inhibited glucose uptake and increased glycogen utilization and TVFA. With rafoxanide no effects were observed. The effects of the drug are explained on the basis of inhibition of ATP synthesis and the inhibition of metabolic flow along the phosphoenol-pyruvate-succinate branch of the respiratory pathway.

For helminths, carbohydrates are the main, if not the only, source of energy, and production of a variety of reduced organic acids as end products is an important feature in the carbohydrate metabolism. Any difference in the carbohydrate metabolism of helminths and their hosts might be usefully exploited in helminth control (Ward, 1982). Previous studies on the energy metabolism of adult *Haemonchus contortus* (Rud., 1803), the most pathogenic gastrointestinal nematode parasite of sheep, goats and other ruminants, have shown that the worm readily utilizes glucose and fixes CO_2, producing propan-1-ol and propionate as the major excretory products (Ward, 1974; Ward and Huskisson, 1978, 1980).

To the best knowledge of ours, the mode of action of anthelmintics affecting biochemical constituents related with carbohydrate utilization and metabolism has not been examined in *H. contortus*. The present studies were undertaken to study the effect of dl-tetramisole (TMS) and rafoxanide (RFX) on total glucose and glycogen contents, and total volatile fatty acids in *H. contortus* under in vitro conditions.

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Table I

Glucose content, glycogen content and amount of total volatile fatty acids

Biochemical constituent	Normal worms collected from goats	Control I Worms incubated for 8 h in Tyrode's solution in vitro	% change from normal
Glucose, mg glucose/g fresh weight	2.32 ± 0.02	3.08 ± 0.02	+24 (S.)
Glycogen, mg glycogen/g fresh weight	11.33 ± 0.12	5.68 ± 0.12	−50 (S.)
Total volatile fatty acids, μmol total volatile fatty acids/50 ml medium	—	3.52 ± 0.12	—

Values are expressed as mean ± SE; n = 4

Materials and methods

Adult worms of *H. contortus* were collected from the abomasums of infected goats *(Capra hircus)* slaughtered locally. The parasites were thoroughly washed in 0.9% saline and processed for further experimentation.

In vitro anthelmintic studies

Concentrations of TMS ranging from 0 to 50 μg/ml were measured into conical flasks each containing 15 worms in 25 ml of Tyrode's solution (Ward, 1974). The flasks were incubated at 37 °C in air-CO_2 (95 : 5). A hundred per cent mortality was achieved with the highest concentration (50 μg/ml) after 10 h. This concentration was, therefore, chosen for subsequent studies. For comparative studies, the same concentration (50 μg/ml) of RFX was used; 100% mortality was achieved after 14 h. The worms were fixed at 8 and at 12 h with TMS and RFX treatment, respectively, i.e. before their expected death. Control I and control II worms were incubated in Tyrode's solution for 8 and 12 h, respectively, and then fixed.

Chemical assays

Glucose content was determined by the method of Nelson (1944), glycogen content by the method of Krisman (1962), and the amount of total volatile fatty acids (TVFA) by the method of Kromann et al. (1967).

Results

The mean and standard error (SE) for glucose, glycogen and TVFA in normal, control and drug-treated *H. contortus* under in vitro conditions are presented in Table I.

in normal, control and drug-treated *Haemonchus contortus* (Rud., 1803)

Worms incubated for 8 h in the presence of TMS	% change from control I	Control II Worms incubated for 12 h in Tyrode's solution in vitro	% change from normal	Worms incubated for 12 h in the presence of RFX	% change from control II
2.75 ± 0.02	−10 (S.)	3.10 ± 0.02	+25 (S.)	3.12 ± 0.02	+0.6 (N.S.)
3.90 ± 0.12	−31 (S.)	5.65 ± 0.12	−50 (S.)	5.62 ± 0.12	−0.5 (N.S.)
1.30 ± 0.16	−63 (S.)	3.81 ± 0.15	—	3.84 ± 0.16	+0.7 (N.S.)

S. = significantly different at $P < 0.05$; N.S. = Non-significant.

After incubation for 8 and 12 h in Tyrode's solution, the glucose content increased by 24% and 25%, and the glycogen content dropped by 50% and 50%, respectively. After treatment with TMS, the glucose content was reduced by 10%, the glycogen content by 31% and the amount of excreted TVFA by 63%. All the three differences are significant. After RFX treatment, no significant change was observed.

Discussion

Adults of *H. contortus* have a readily available and constant source of glucose from the blood and the wall of abomasum of sheep (Ward, 1974). Previous studies on the carbohydrate metabolism of adult *H. contortus* (Ward and Huskisson, 1978, 1980) and the presence of most of the enzymes of the glycolytic pathway (Kaur and Sood, 1982) have shown that the energy demand of the worm depends almost entirely on glucose catabolism.

It is generally accepted that the glucose pool is relatively low when the parasites are removed from the host, and during their in vitro incubation in the presence of glucose, internal glucose concentrations increase significantly as the parasite equilibrates with the medium (Cornish and Bryant, 1976). These findings accord well with the results drawn from the present study that upon in vitro incubation in the presence of glucose, glucose concentration increases. The increased glucose pool in itself does not mean an increased flux through the system (Cornish and Bryant, 1976). However, in the present work, an increased glucose content was associated with a significantly decreased glycogen content. These findings are similar to those observed in *Fasciola hepatica* (Threadgold and Arme, 1974). The drop in glycogen levels may probably be due to initial expulsion of eggs or, possibly, to the higher rate of glycogen degradation than that of glucose utilization.

All these changes do not follow an increased flux through the system and this imbalance is most likely associated with the increase in the amount of TVFA excreted. A similar situation was observed in *Ascaris* incubated in a bacteria-free glucose medium (Epps et al., 1950).

Van den Bossche (1972) has shown that in *Ascaris* and in some other nematodes, mebendazole inhibits glucose uptake and depletes glycogen reserves. These effects, he suggests, are ultimately responsible for lowering ATP levels within the parasites. Our studies have shown similar observations in *H. contortus* after TMS treatment. Therefore, as in *Ascaris* (Beames, 1971), glucose uptake in *H. contortus* is an active process. Raj and Kurup (1967) observed decreased glucose and glycogen contents after treatment of *Ascaris* with palasonin. However, no significant effect on the glycogen content was observed when *Ascaridia galli* was exposed to TMS treatments (Chakraborty et al., 1976). Even though uptake of glucose is inhibited in the presence of TMS, the carbon flux is maintained and production of TVFA declines. Van den Bossche and Janssen (1967) observed a similar situation in *Ascaris*.

Anaerobically, the phosphoenolpyruvate-succinate pathway is the major source of ATP. The effects of the drug can, therefore, be explained in terms of inhibition of ATP synthesis. The drug might impair the energy status of the parasite, and ATP levels as well as total nucleotides decrease. As ATP levels fall, there will be a balance between the stimulation of metabolic flux due to the removal of allosteric inhibition of regulatory enzymes such as hexokinase, phosphofructokinase, pyruvate kinase and phosphoenolpyruvate-carboxykinase (Rahman and Bryant, 1977).

The present study has indicated effects of TMS on some biochemical constituents related with carbohydrate utilization and metabolism. It is, however, difficult to tell whether the effects initiate one another, or whether they are exerted simultaneously. Thus, the inhibition of glucose uptake may be a consequence of diminished ATP levels or may be a direct effect of the drug. It is, therefore, probable that an early effect of the drug is on the phosphorylation of ADP and on the turnover of nucleoside phosphates.

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ANTHELMINTIC STUDIES ON *SETARIA CERVI*: HISTOCHEMICAL ALTERATIONS IN GLUCOSE-6-PHOSPHATASE, ADENOSINE TRIPHOSPHATASE AND MALIC DEHYDROGENASE

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The effect of the anthelmintics diethylcarbamazine citrate (DEC), tetramisole and levamisole on the enzymatic activity of *Setaria cervi* was demonstrated histochemically. Glucose-6-phosphatase, malic dehydrogenase, and adenosine triphosphatase were found to be normally localized in the cuticle, subcuticle, fibrillar part of muscles, oesophagus, intestine, boundary walls of vagina and uteri, developing embryos, chords and microfilariae parasitizing untreated control rats. The activities of these enzymes were found to be appreciably decreased in the worms parasitizing drug-treated rats.

Glucose-6-phosphatase (G-6-Pase), adenosine triphosphatase (ATPase) and malic dehydrogenase are important enzymes of parasitic nematodes. The biological significance of these enzymes in the functional integrity of the worms has been established beyond doubt. The normal activities of these enzymes have been reported to be interrupted by the use of certain anthelmintics such as dithiazanine, suramin and levamisole (Bueding et al., 1961; Van den Bossche, 1972; Anwar et al., 1978). However, it is not clear whether the observed biochemical or histochemical changes are the primary cause or result from the functional damage produced by the drugs.

The present paper deals with the histochemical alterations in G-6-Pase, ATPase and malic dehydrogenase in *Setaria cervi* following treatment with diethylcarbamazine citrate (DEC), tetramisole or levamisole. The chemotherapeutic efficacy of these drugs has earlier been proved against *Setaria cervi* (Baqui and Ansari, 1976).

Materials and methods

Twenty laboratory-bred white rats *(Rattus norvegicus)*, weighing 100–125 g, were used. Live *Setaria cervi* worms, collected from the freshly slaughtered buffaloes at the local abattoir, were transplanted intraperitoneally into white rats (Khatoon and Baqui, 1981). The infected rats were divided into four groups. Groups I, II and III were treated with levamisole, tetramisole and DEC, respectively, from the second day following infection. Levamisole

and tetramisole were given orally at the dosage of 15 mg/kg/day, whereas DEC at the dosage of 100 mg/kg/day, on 5 consecutive days. Group IV served as untreated control. Rats of all the four groups were autopsied on the 6th day when the treatment was over. Treated and untreated worms, recovered on autopsy, were fixed in various fixatives and processed histochemically for the localization of G-6-Pase, ATPase and malic dehydrogenase according to the methods of Pearse (1960).

Results

All the drugs used in the present experiment were found to be effective and showed inhibitory effect in respect of these enzymes (G-6-Pase, ATPase and malic dehydrogenase) of *Setaria cervi*. However, tetramisole and levamisole

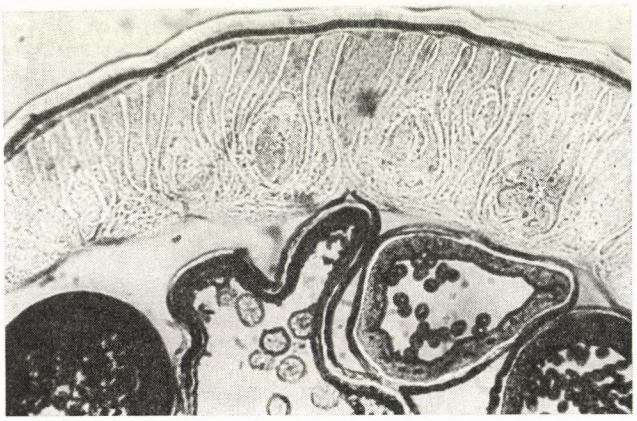

Fig. 1. Section of the control worm *(Setaria cervi)*. G-6-Pase activity in the subcuticle, intestine, uterine wall and developing embryos. ×400

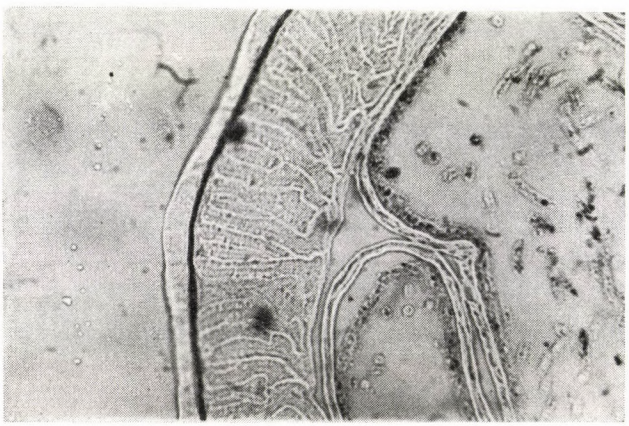

Fig. 2. Section of a female worm showing decreased G-6-Pase activity in the subcuticle, uterine wall and developing embryos, following tetramisole treatment. ×400

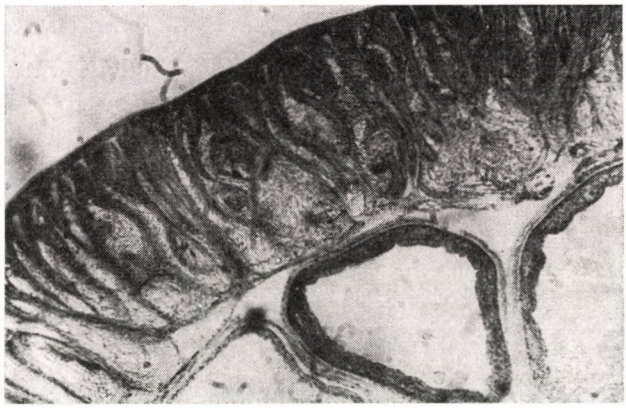

Fig. 3. Section of the control worm. Malic dehydrogenase activity in the fibrillar part of muscle and uterine wall. ×400

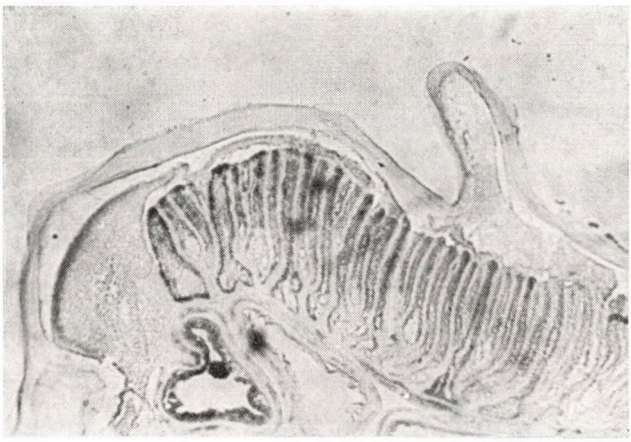

Fig. 4. Section of a worm showing decreased malic dehydrogenase activity in the subcuticle, musculature and uterine wall following levamisole treatment. ×400

were found to be more effective than DEC. Figs 1-6 show the localization of these enzymes in the untreated control worms and the inhibitory effects of the drugs in respect of these enzymes.

G-6-Pase activity was noted in the subcuticle, oesophagus, intestine, boundary wall of vagina and uteri, developing embryos, microfilariae and chords in the untreated worms (Fig. 1). Appreciable decrease in its activity was noticed in the tetramisole-treated worms. The oesophagus, intestine, boundary walls of vagina and uteri, developing embryos and microfilariae showed marked reduction of G-6-Pase activity. However, no change in the enzyme activity was noted in the subcuticle (Fig. 2).

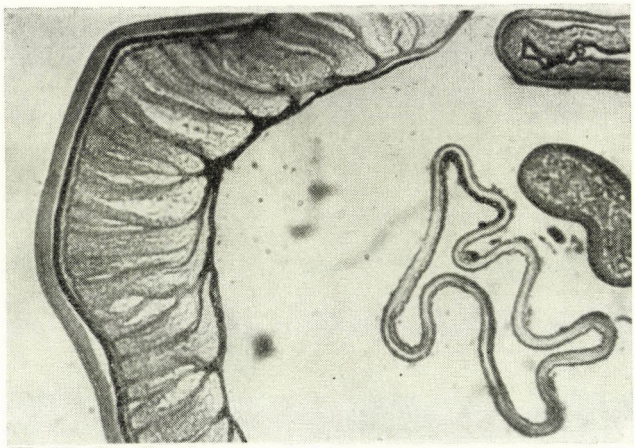

Fig. 5. Section of the control worm. ATPase activity in the subcuticle, muscle tails, intestine and uterine wall. ×400

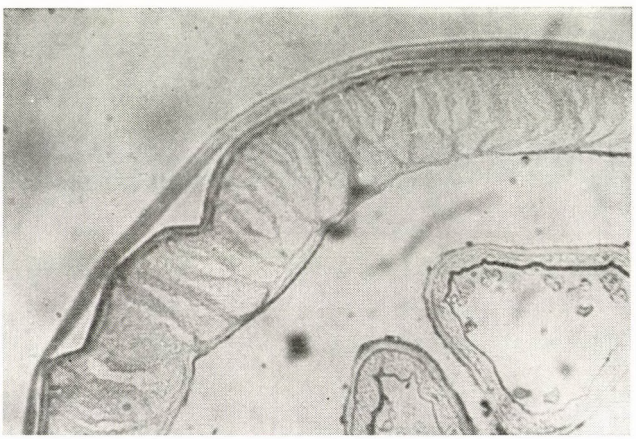

Fig. 6. Section of a worm showing decreased ATPase activity in the subcuticle, musculature and uterine wall following DEC treatment. ×400

Malic dehydrogenase activity was localized in the subcuticle, the fibrillar part of the muscles, oesophagus, boundary walls of uteri and vagina (Fig. 3). The enzyme activity was reduced in the worms treated with levamisole (Fig. 4).

ATPase activity was found to be normally localized in the subcuticle, muscle tails, chords, boundary walls of the vagina and uteri, developing embryos and microfilariae (Fig. 5). The activity of this enzyme was found to be decreased in all these organs of the worms (Fig. 6) following DEC treatment.

Discussion

Earlier studies have shown that tetramisole, levamisole and DEC cause adverse effects on the adult worms and microfilariae of *Setaria cervi*, which are characterized by the complete disappearance of microfilariae, and immobility, early degeneration and irreversible paralysis of the worm (Baqui and Ansari, 1976; Khatoon and Baqui, 1981).

Evidence is available which indicates that several antiparasitic drugs exert their primary action by interfering with the normal metabolic activities of parasites, especially with some phase of the glycolytic and/or oxidative metabolism of parasites (Van den Bossche, 1976).

Active anthelmintic compounds have been reported to interfere with the carbohydrate metabolism at different levels, especially with the absorption of carbohydrates and their intracellular utilization (Von Brand, 1966). According to Von Brand (1966), interference with the carbohydrate metabolism by antiparasitic drugs is not surprising in view of the enormous importance of carbohydrates in the metabolism of the parasites. In vitro studies of Anwar et al. (1978) have revealed that glucose uptake and glycogen synthesis are considerably decreased in *Litomosoides carinii* and *Setaria cervi* following treatment with levamisole and DEC. However, Walter (1979), Walter and Schulz-Key (1980) and Khatoon et al. (1982) have reported a decrease in lactate, malate and succinate dehydrogenase activities in *Dirofilaria immitis*, *Onchocerca volvulus* and *Setaria cervi* following treatment with suramin, DEC, tetramisole and levamisole. In our investigations the activities of the three examined enzymes have been found to be decreased in treated worms.

Von Brand (1966) has contended that many anthelmintics are not specific in the sense that they just inhibit a single enzyme system but attack a number of other components also. A somewhat similar line of reasoning can be employed to prove the corresponding mechanism in the case of the anthelmintic activities of DEC, levamisole and tetramisole against *Setaria cervi*. The earlier investigations have revealed that the drugs also inhibit the acid and alkaline phosphatase and cholinesterase activities of *Setaria cervi*, a filariid of buffaloes (Khatoon and Baqui, 1982; Baqui and Khatoon, 1982).

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ACTA VETERINARIA

ТОМ. 30.—ВЫП. 4.

РЕЗЮМЕ

ВТОРИЧНЫЙ АМИЛОИДОЗ ПОЧЕК У СОБАКИ И ЕГО ПОСЛЕДСТВИЯ

М. ДОБОШ-КОВАЧ, ДЬ. ДЕАК и ЛИЛИЯНА БАРТАЛИЧ

На основании патологоанатомических исследований авторами докладывается о спонтанном амилоидозе почек у 5 собак. У двух собак амилоидоз почек сопровождался нефрозом, у двух собак в качестве осложнения к нефрозу присоединились тромбоз легочной артерии, легочных вен и задней полой вены, в одном случае цирроз почек при осложнении продолжительной уремии. Гистологическими исследованиями доказано, что во всех пяти случаях налицевствовал вторичный амилоидоз почек.

В дискуссии обсуждают возможный патомеханизм возникновения склонности к тромбозу, присоединяющегося в качестве осложнения к синдрому нефроза.

ЧИСТКА И ЧАСТИЧНАЯ ХАРАКТЕРИСТИКА ИНГИБИТОРА ПРОТЕАЗЫ

Ш. ЮХАС

Автором изучен в клеточном экстракте из *Proteus vulgaris* фактор, тормозящий активность трипсина и химотрипсина. Ингибитор очищался аффинитет-хроматографическим путем. Энзимной предварительной обработкой доказано, что тормозящий эффект двух энзимов обусловливается тем же (или очень близкими друг другу) центром(ами). Молекулярный вес ингибитора, определенный хроматографией на геле, равняется 30 000—32 000.

ИЗМЕНЕНИЯ КОНЦЕНТРАЦИИ ГОРМОНОВ ЩИТОВИДНОЙ ЖЕЛЕЗЫ И ПОЛОВЫХ СТЕРОИДОВ В СЫВОРОТКЕ ПЕТУХОВ И КУР ПРИ ФОРСИРОВАННОЙ ЛИНЬКЕ

ДЬ. ПЕТЕШ, З. СЕЛЕНИ и П. ПЕЦЕЛИ

Изучались изменения функции щитовидной железы и половых желез во время форсированной линьки, вызванной путем ограничения корма, питьевой воды и света, дальше, при помощи понижения температуры. У куры концентрация прогестерона в сыворотке во время линьки математически достоверно повышается, потом беспрерывно понижается, тогда как 17-β-эстрадиол во время данного процесса находится на низком уровне. Тироксин у куры сначала вместе с прогестероном повышается, во время линьки остается на высоком уровне, а уже у оперившихся животных показывает снова низкий уровень в сыворотке. Трийодтиронин в начальном периоде понижается, а у оперившихся животных снова повышается.

У петухов в начале линьки концентрация тестостерона понижается и в течение всего периода остается на низком уровне. Параллельно с падением уровня тестостерона повышается уровень тироксина. Трийодтиронин подобно описанию у кур — но математически не достоверно — изменяется.

Согласно полученным данным у куры и петуха в условиях форсированной линьки смена регулируется изменением взаимодействия щитовидной железы и половых желез.

Повышение концентраций прогестерона и тироксина у куры, действуя на филликулы, вызывает потерю перьев. У петуха повышение концентрации тироксина и его действие на перьевые фолликулы обеспечивается низким уровнем тестостерона.

Во время роста новых перьев у животных обоих полов как тироксин, так и трийодтиронин показывают высокую концентрацию в сыворотке, тогда как таковая стероидов половых желез является низкой.

МЕТАБОЛИЗМ ВОДЫ И ЭЛЕКТРОЛИТОВ У ОВЦЫ

Б. ЮХАС, Б. СЕГЕДИ и М. КЕРЕСТЕШ

В трех сериях экспериментов авторами изучалось действие нагрузки повареной солью и водой на метаболизм электролитов и воды у овец, снабженных фистулой в рубец и катетерами в околоушную слюнную железу, в мочевод и яремную вену. Животных заранее снабжали разным количеством повареной соли и воды. В исследованиях применялся изотоп ^{24}Na.

Обнаружено, что при недостаточном снабжении повареной солью концентрация в слюне Na^+ существенно понизилась, а таковая K^+ резко повысилась. Это и было причиной резкого понижения частного Na^+/K^+. При достаточном снабжении повареной солью ^{24}Na появлялся в четыре раза быстрее, чем при недостаточном снабжении ею.

Обнаружена тесная взаимосвязь между выделением слюны и таковым мочи. При понижении снабжения повареной солью наступило уменьшение диуреза и выделения слюны и, подобно этому, понижение выделения Na^+ и K^+ слюной и мочей. При нагрузке повареной солью повысилось выделение мочи, но не было изменения в выделении слюны. Большое количество Na^+ и K^+ выделялось мочей. Соответственно этому нагрузка водой изменила объем жидкости рубца и ее осмотического давления. Это вело к изменению в диурезе и выделении слюны и в количестве выделяемых мочей и слюной Na^+ и K^+. На основании полученных данных можно заключить, что изменение осмотического давления жидкости рубца очень быстро и резко сказывается на метаболизме воды и электролитов у жвачных.

ДЕЙСТВИЕ ПОЛОВЫХ СТЕРОИДОВ НА ТРАНСПОРТ ЛИПИДОВ И В ЛИПИДАХ РАСТВОРЯЮЩИХСЯ ВИТАМИНОВ У КУРЫ

ДЬ. ЛЕНЧЕШ и М. МЕЗЕШ

Действие половых стероидов — 17-β-эстрадиола и его комбинаций с прогестероном и/или тестостероном — изучено на уровень всего липида, каротина и растворяющихся в липидах витаминов А и Е в крови кур мимо сезона яйцекладки. Во время четырех недель обработки животных стероидами уровень этих препаратов в сыворотке определяли еженедельно.

Обнаружено, что количество всего липида систематически повышалось в группах кур, обработанных эстрадиолем и эстрадиол + прогестероном. Уровень каротина не показывал математически достоверных изменений. Содержание витамина Е в сыворотке математически достоверно повысилось в группах кур, получавших эстрадиол, на первой неделе обработки. Ретиниловый эстер наивысшую концентрацию показывал на второй неделе обработки эстрадиолем. Изменения уровня ретинола были подобными таковым витамина Е.

ОПРЕДЕЛЕНИЕ НЕКОТОРЫХ НОРМАЛЬНЫХ ПАРАМЕТРОВ В КРОВИ ЭМБРИОНОВ КУРЫ, ФАЗАНА И ПЕРЕПЕЛА

Л. ВАРНАДЬ

В работе представлены показатели щелочной фосфатазы, неорганического Са и Р, гематокрита и количества эритроцитов в крови эмбрионов куры, фазана и перепела непосредственно перед вылуплением. При помощи описанного простого способа получения крови и соответствующих лабораторно-диагностических и биохимических методов есть возможность определения ряда параметров крови и сыворотки эмбриона.

На основании многочисленных исследований в данной работе автор приводит основные показатели, которые до сих пор в литературе нигде не были представлены. Некоторые нормальные показатели могут быть полезными для тех, кто занимаются вопросами эмбриологии, тератологии на эмбрионах птиц.

ДЕЙСТВИЕ НАРУЖНЫХ ФАКТОРОВ ПЕРЕД УБОЕМ НА АКТИВНОСТЬ ЭНЗИМОВ КРЕАТИНИН-ФОСФОКИНАЗЫ (КФК) И ЛАКТАЗА-ДЕГИДРОГЕНАЗЫ (ЛДГ) У СВИНЬИ

М. СИЛАДИ, М. ВИТТМАНН, Ф. ГУБА и Л. ВИГ

Авторами изучалась активность энзимов КФК и ЛДГ после бережного транспорта из малого расстояния траспортированных (35 км) и убитых свиней и таковых подвергавшихся после транспорта моциону на расстоянии 350 м. Животные были гибридными или простого скрещевания. Исследования проводились в летнюю, осеннюю и зимнюю поры года.

Обнаружено, что разные поры года по разному сказывались на указанных параметрах крови. Наименьшая активность, то есть наиболее приятные параметры крови получены осенью именно в октябре месяце, а наиболее плохие, то есть наиболее высокие, в январе-феврале месяцах.

На неблагоприятное действие в группе показывали не только средние показатели, но оно проявлялось и в том, что энзимная активность у некоторых особей была особенно высокой.

Моцион между транспортом и убоем, как добавочная нагрузка, не отразилась на изученных параметрах.

Параметры крови свиней, полученные в те же поры года и при таких же условиях не отличались друг от друга, таким образом разницу в чувствительности к данному стрессору не удалось выявить.

ИССЛЕДОВАНИЯ ПО ЭНЗИМНОЙ АКТИВНОСТИ ПЛАЗМЫ СЕМЕНИ БЫКОВ ДЛЯ КОНТРОЛЯ ПОДВИЖНОСТИ, ОПЛОДОТВОРЯЮЩЕЙ СПОСОБНОСТИ И ЗАМОРАЖИВАЕМОСТИ ЖИВЧИКОВ

М. А. Р. ИБРАГИМ

Сорок четыре быка венгерской пестрой породы, использующихся в искусственном осеменении, разделены в две группы, согласно их воспроизводительных данных (картина семени, ее замораживаемость, оплодотворяющая способность). Изучалась активность энзимов семени, как то, глутамин-пируват трансаминазы (ГПТ), глутамин-щавелевокислой трансаминазы (ГЩТ) и гиалуронидазы и данные сравнены с картиной семени. Активности ГЩТ и ГПТ измерялись, тогда как гиалуронидаза определялась иммунодиффузионным методом при помощи анти-гиолуронидазной сыворотки от кролика. Показатели всех трех энзимов плазмы семени были более высокими у быков слабшей воспроизодительной способности.

МОРФОЛОГИЧЕСКИЕ ИЗМЕНЕНИЯ ЖИВЧИКОВ БЫКА ПРИ ЗАМОРОЖЕНИИ И РАСТАЯНИИ

М. А. Р. ИБРАГИМ, Л. КОВАЧ и Б. Л. ТОТ

Лучшее понимание физиологии, патофизиологии и морфологии полового тракта и структуры живчиков обеспечивает лучшие шансы на диагносцирование и лечение дисфункций этого органа и рациональное основание на изучение новых подходов лечения бесплодия самцов.

Данному исследованию подвержено сто быков производителей венгерской пестрой породы. Изучение картины акросомы осуществлялось бальным методом 0 – 3 при разном состоянии семени: А) свежее семя без обработки; Б) разбавленное и уравновешенное семя на 3,5 часа; В) замороженное при — 196°С и растаянное семя в течение часа при +46°С; Д) замороженное при — 196°С, хранившееся в таком состоянии в течение одних суток и потом растаянное семя.

Обнаружено, что количество дефектных живчиков и таковых с дисфункцией акросомы в состоянии заморожения повышается. Соотношение между картиной акросомы, подвижностью и оплодотворяющей способностью как незамороженных, так и заморожен-

ных живчиков, было положительным. Структура акросомы, это новый, световым микроскопом получаемый критерий, при помощи которого просто и быстро, балльным методом можно оценить в рутинной работе семя, поведение живчиков, их замораживаемость и оплодотворяющую способность. Это аккуратный и чувствительный параметр надежного определения нормального состояния клеток.

ДЕЙСТВИЕ ГЛУБОКОГО ЗАМОРОЖЕНИЯ НА СПЕРМУ ХРЯКА

М. А. Р. ИБРАГИМ и Л. КОВАЧ

Действие глубокого замораживания изучено на сперме 50 хряков производителей пород дюрок, эстонской, низменной ГДР и германской поясничной. Сперму получали ручным методом. В племенный период 1979-ого года из 190 эякулятов приготовлено 1218 доз глубоко-замороженной спермы хряков в специальных резиновых тюбиках (искусственных соломинках). При глубоком замораживании в качестве разбавителя пользовались 11%-ным раствором лактозы к которому добавляли 0,1% порошка снятого молока и криопротективные вещества, как то, глицерин и паста Орвус Ес. Глубоко-замороженную сперму растаяли на водяной бане температуры 52°С в течение 52 секунд. Потом сперму нагревали до 20°С и разбавляли тающей жидкостью (Beltsville или OLEP Liquid и проводили осеменение свиноматок. Супоросность свиноматок определяли ультразвуком 40 дней спустя после осеменения. Среди 397 осемененных глубоко-замороженной спермой свиноматок 184 (46,3%) опоросилось при средней величине пометов в 9 поросят. Морфология головки живчиков до и после глубоко замораживания изучалась световым и электронным микроскопом. Математически достоверные разницы в морфологии обнаружены согласно возрасту и породы быков.

СРАВНИТЕЛЬНЫЕ БИОХИМИЧЕСКИЕ И ПАТОЛОГИЧЕСКИЕ ИССЛЕДОВАНИЯ ПО ОСТРОМУ АЦИДОЗУ У БУЙВОЛЯТ ОТ КОРМЛЕНИЯ МЕЛАССОЙ И ЗЕРНОМ

С. С. РАНДАВА, Л. Н. ДАС и С. К. МИСРА

Буйволятам раз скормили мелассу (группа 1) или пшеничную крупу (группа 2). У животных группы 1 наступило временное повышение концентрации молочной кислоты в содержимом рубца и в крови, тогда как у буйволят, получивших пшеничную крупу, 2—3-хкратное повышение концентрации молочной кислоты было продолжительным. Скормление мелассы вызвало гипогликемию, а пшеница — гипергликемию. С этими изменениями биохимических параметров совпадали отклонения в интенсивности и качестве патогистологических изменений мозга, экзокринной и эндокринной частей поджелудочной железы, рубца, сетки и печени. Патогистологические изменения были в корреляции с биохимическими таковыми.

HAEMONCHUS CONTORTUS: ДЕЙСТВИЕ АНТГЕЛЬМИНТИКОВ НА ОБЩЕЕ СОДЕРЖАНИЕ ГЛЮКОЗЫ, ГЛИКОГЕНА И СИВУЧИХ ЖИРНЫХ КИСЛОТ

РАНБИР КАУР и М. Л. СООД

Изучалось действие 50 μг/мл DL-тетрамизола и рафоксанида на половозрелых особей *Haemonchus contortus* in vitro после инкубации 8 и 12 часов соответственно. При инкубации в растворе Тайрода без антгельминтиков содержание глюкозы и гликогена повысились, а таковое сивучих жирных кислот понизилось. Причиной понижения уровня гликогена может быть начальное выделение яиц или более энергичное расщепление его, а не степень использования глюкозы.

Тетрамизол тормозил прием глюкозы и повысил использование гликогена и сивучих жирных кислот. От рафоксанида не получено этого эффекта. Эффект действия антгельминтика объясняется на базе торможения синтеза АТР и торможения метаболических отправлений в направлении фосфоэнолпируват-сукцинатной ветви.

АНТГЕЛЬМИНТОЛОГИЧЕСКИЕ ИССЛЕДОВАНИЯ НА *SETARIA CERVI*: ГИСТОХИМИЧЕСКИЕ ИЗМЕНЕНИЯ ГЛЮКОЗА-6-ФОСФАТАЗЫ, АДЕНОЗИН ТРИФОСФАТАЗЫ И ЯБЛОЧНОКИСЛОЙ ДЕГИДРОГЕНАЗЫ

Х. КАТОН, ВАЙИХУЛЛАХ, А. БАКИ и Я. А. АНСАРИ

Гистохимически изучено действие нескольких антгельминтиков, как то, лимоннокислого диэтилкарбамазина (ЛДЭК), тетрамизола и левамизола на активность некоторых энзимов у *Setaria cervi*. В нормальных условиях глюкоза-6-фосфатаза, аденозин трифосфатаза и яблочнокислая дегидрогеназа локализуются в кутикуле, субкутикуле, фибриллярной части мышц, пищеводе, кишечнике, стенке вагины на переходе в матку, развивающихся эмбрионах, хордах и микрофиляриях. Активность перечисленных энзимов после обработки нематоды указанными антгельминтиками заметно ослабла.

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