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FEEDING ANIMAL FATS TO SHEEP

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In an experiment lasting 7 weeks, 18% of the calculated energy requirement of 5 nonpregnant ewes was met by giving natural fats of animal origin mixed in the ration (at a level of 6.6%).

Changes in certain blood and blood plasma parameters of lipid and energy metabolism were monitored at 5 time-points during the experiment. Samples of rumen fluid were taken three times for determining the concentrations of volatile fatty acids (VFA). Rumen fermentation was studied by *in sacco* method. Total lipid (TL) content of the liver and fatty acid composition of the liver tissue and subcutaneous adipose tissue (from the tailhead) were analyzed at the beginning and at the end of the experiment.

At the concentration used in this experiment, fat supplementation caused neither digestive disturbances nor any other adverse changes in the animals' health status. It did, however, exert a significant influence on blood plasma lipid composition. TL and total cholesterol (TCh) concentration increased and blood glucose level decreased. A rise in triglyceride (TG) content was accompanied by a drop in free fatty acid (FFA) concentration.

The *in sacco* experiments and volatile fatty acid (VFA) levels in the rumen fluid suggested an impaired crude fibre digestion in the rumen. At the same time, fat supplementation enhanced rumen proteolysis.

The TL content of liver samples did not exceed the physiological limit. The liver biopsy samples had decreased myristic acid and increased stearic and oleic acid concentrations.

No change occurred in the fatty acid composition of the fat depots.

Key words: Fat supplementation, animal fat, lipid metabolism, sheep, ruminal digestion and/or fermentation

The need to meet the enormously increased energy demand of high-producing dairy cows has called for new solutions entirely different from the feeding practices used earlier. Studies have been done to test whether dietary fats of animal origin could be used for that purpose.

Most monogastric animals are known to utilize fat, the nutrient richest in energy, very well. On the other hand, the digestive physiological characteristics of herbivorous animals, particularly ruminants, restrict the utilization of that nutrient. Fat is not a natural feed for ruminants.

Although fats are efficiently absorbed from the intestine of ruminants (Andrews and Lewis, 1970; Steele and Moore, 1968), their presence in the rumen may impair the efficiency of fermentation. The digestibility of crude fibre decreases as natural fats "enwrap" (coat) the feed particles present in the rumen fluid and make them hardly accessible to bacteria (Rohr et al., 1978).

The shift occurring in volatile fatty acid (VFA) production towards propionic acid seems to be energetically favourable (Hagemeister and Kaufmann, 1979; Mattos and Palmquist, 1974; Rohr et al., 1978). At the same time, the presence of fats in the rumen involves some adverse effects, e.g. polyunsaturated fatty acids inhibit the activity of cellulolytic microorganisms (Henderson, 1973; Maczulak et al., 1981), and crude protein degradability in the rumen may decrease (Rohr et al., 1978).

In view of the above facts, dietary fats are recommended for ruminants between 2 and 5% of the dry matter of the feed (Kirchgessner, 1982; N.C.R., 1978). Certain experimental data, however, seem to indicate that although dietary fat given at levels higher than 5% may positively affect the metabolism of ruminants, it is favourable in respect of their energy supply (Palmquist and Conrad, 1978; Smith et al., 1978; Kronfeld et al., 1980; Magdus et al., 1988).

The importance of fat supplementation and the contradictory experimental results obtained in this subject have prompted us to conduct further experiments on feeding animal fats to sheep as a model animal. The objective of these experiments was to determine the effect of animal fats on rumen fermentation, on certain parameters of lipid metabolism, particularly the lipid fractions of the blood plasma, and on the fatty acid composition of the liver and the subcutaneous adipose tissue.

Materials and methods

Five nonpregnant Merino ewes (body mass: 48–50 kg) were used in the experiment. The animals were kept in individual pens and their ration consisted of concentrate feed mixture and alfalfa hay according to their calculated requirement. The daily ration was given in two parts. Three weeks before the beginning of the experiment the ewes were fitted with a permanent rumen fistula which permitted continuous sampling of rumen contents and fermentation experiments.

The experiment was self-controlled and consisted of three phases (Fig. 1). In the first phase *(preliminary phase*, which lasted 7 days, the sheep were given a normal ruminant basal ration without any fat supplementation (Table 1). The results obtained for samples taken in that phase (control samples) served as a basis for comparison.

In the second phase *(adaptation phase*, which lasted 14 days and served for adapting the animals to the experimental diet, a ration meeting the animals' calculated requirements was fed. In that ration, 6.6% of the energy content was met by fat of animal origin (Favorit-40, a feed additive containing 40% animal fat and 60% flaked corn; produced by Monor State Farm, Hungary).

Table 1

Composition and nutrient contents of the experimental diet

Constituents	Basal	Adaptation	Experimental
		diet	
Fodder mix (g)	1000	1000	1000
Corn meal	768	686	603
Extr. sunflower meal	190	190	190
Favorit-40	—	82	165
Mineral and vitamin premix	42	42	42
Alfalfa hay (g)	300	300	300
Dry matter (g)	885	889	894
Crude protein (g)	136	135	134
Ether extract (g)	33	60	86
Crude fibre (g)	45	47	51
Energy (MJ NE_m)	12,624		

Table 2

Fatty acid composition of the dietary fat supplement (Favorit-40) expressed in per cent of total fatty acids

Fatty acid	Designation	%
Lauric acid	$C_{12:0}$	0.09
Myristic acid	$C_{14:0}$	1.40
Palmitic acid	$C_{16:0}$	25.83
Palmitoleic acid	$C_{16:1}$	2.83
Margaric acid	$C_{17:0}$	0.58
Stearic acid	$C_{18:0}$	10.17
Oleic acid	$C_{18:1}$	43.52
Linoleic acid	$C_{18:2}$	13.17
Linolenic acid	$C_{18:3}$	1.04
Arachic acid	$C_{20:0}$	0.20
Behenic acid	$C_{20:1}$	1.11
Other fatty acids		0.06
Saturated fatty acids/unsaturated fatty acids = 40 : 60		

In the third phase *(experimental phase)* the fat content of the ration was increased in such a way that 18.0% of the calculated energy content was met by giving dietary fat of animal origin (Favorit-40). The experimental phase lasted 49 days.

The composition of rations fed in the different phases of the experiment is shown in Table 1. The fatty acid composition of fat given as supplementation is presented in Table 2.

The types and times of sampling throughout the experiment are given in Fig. 1.

Basal ration	Adaptatior phase			Experimental feed	
Preliminary phase	Adaptation phase			Experimental phase	
0	7	14	21	35	70 days
	1	2	3	4	5
	BRLAI	IB	IBR	IB	BRLAI

Fig. 1. Design of the experiment and sampling times. 1–5: sampling times; B = blood sample; R = rumen content; L = liver biopsy; A = adipose tissue biopsy; I = *in sacco* experiment

Blood samples were collected on five occasions: a control sample was taken at the end of the preliminary stage (time-point 1), and further blood samplings took place in the middle (time-point 2) and at the end (time-point 3) of the transition phase, and then in the middle (time-point 4) and at the end (time-point 5) of the experimental phase. Blood sampling was done immediately before the morning feeding in all cases.

Blood samples were assayed for glucose (G) concentration (Trinder, 1969), and blood plasma samples for total lipid (TL; Frings and Dunn, 1970), triglyceride (TG; Biggs et al., 1975), free fatty acid (FFA; Duncombe, 1964), total cholesterol (TCh; Watson, 1960) and urea (U; Fawcett and Scott, 1960) concentration as well as aspartate aminotransferase (AST; Haschen, 1969) activity.

For determining the volatile fatty acid (VFA) content of the rumen fluid, 50 ml samples were collected through the permanent rumen fistula, always from the same place, at sampling times 1, 3 and 5, before the morning feeding. The VFA content of the samples was determined by gas chromatography (Husvéth and Gaál, 1988).

Rumen fermentative activity was studied by an *in sacco* method (Mehrez and Orskov, 1977; De Boever et al., 1984). For this method, 17×19 cm polyester sacs of 50 μ pore size, containing 2500 pores per cm^2, were prepared. Three g finely chopped, homogeneous alfalfa hay was measured into the sacs, then the sacs were tied down with a strong nylon string the end of which was fastened to the outer end of the fistula after it had been introduced into the rumen. For each test, 3 sacs containing the same sample were placed into the rumen and incubated *in situ* for 48 h. Each test was repeated on two successive days. After incubation, the sacs were removed from the rumen and washed in running tap water until the effluent became clear. Subsequently, the sacs were dried in a drying oven at 60 °C, weighed and the residues contained by

them were tested for crude protein and crude fibre content (MSz-6830, 1983). The degradation of the two nutrients in the rumen was calculated as the difference between the original alfalfa sample and the residues in crude protein and crude fibre content.

To detect possible changes in the fatty acid composition of the fat depots, adipose tissue samples were taken from the animals at the beginning and at the end of the experiment (at time-points 1 and 5). Samples were taken from the subcutaneous adipose tissue around the tailhead, from an about palm-sized area at the mid-point of the line connecting the point of hip and the tailhead. About 1 g adipose tissue was excised from that area (Johnson et al., 1977).

Simultaneously with collecting the adipose tissue samples, 150 to 200 mg samples were taken from the liver by percutaneous needle biopsy to determine total lipid content and fatty acid composition of lipids.

The lipid content of adipose tissue and liver samples was extracted by the method of Folch (1957). After extraction, the lipid content of the liver samples was measured gravimetrically. The fatty acid composition of the extracted lipids was determined by gas chromatography (Husvéth et al., 1982).

The results were analyzed by statistical methods (Sváb, 1981). Besides calculating means and standard deviations, a two-sample *t* test was performed.

Results

Analysis of blood samples

The G, TL, TCh, FFA, TG and U concentrations and AST activity of the blood samples in the different phases of the experiment are presented in Table 3.

Blood glucose level was physiological at the beginning of fat feeding (2.69 mmol/l), then showed a transient 25% decrease ($p < 0.05$). Subsequently it again came close to the baseline value (2.96 mmol/l) and decreased again by the end of the experiment (2.06 mmol/l).

An opposite tendency could be observed in *blood plasma TL content.* Its initial value (1.52 g/l) rose almost to double by the end of the adaptation phase ($p < 0.05$), then, after a transient slight decrease, by the end of the experiment it reached a value (2.75 mmol/l) significantly ($p < 0.001$) higher than the baseline value.

Blood plasma TCh concentration underwent a significant rise ($p < 0.01$) already during the transition phase. By the end of the adaptation phase (3rd sampling) plasma TCh level (2.19 mmol/l) was twice the basic value. By the 4th blood sampling plasma TCh decreased to 1.39 mmol/l, but at the end of the experiment it was more than twice the control value (2.26 mmol/l).

Table 3

Changes in blood glucose (G), blood plasma total lipid (TL), total cholesterol (TCh), free fatty acid (FFA), triglyceride (Tg) and urea (U) concentrations and AST activity in different phases of the experiment

		1	2	3	4	5		
G, mmol/l	$\bar{x}$	$2.69^{a,b,c}$	1.86^{a}	1.96^{b}	2.96	2.06^{c}	a, b	$p < 0.01$
	$\pm s$	0.08	0.29	0.28	0.37	0.44	c	$p < 0.001$
TL, g/l	$\bar{x}$	$1.52^{a,b}$	1.78	2.84^{b}	2.42	2.75^{a}	a	$p < 0.001$
	$\pm$SD	0.34	0.22	0.55	0.45	0.62	b	$p < 0.05$
TCh, mmol/l	$\bar{x}$	$1.08^{a,b,c}$	1.79^{b}	2.19^{c}	1.39	2.26^{a}	a, c	$p < 0.001$
	$\pm$SD	0.18	0.15	0.36	0.41	0.68	b	$p < 0.01$
FFA, mmol/l	$\bar{x}$	0.184^{a}	0.216^{b}	0.188^{c}	$0.140^{d,c}$	0.060^{a}	a	$p < 0.01$
	$\pm$SD	0.070	0.003	0.016	0.040	0.030	b, c	$p < 0.05$
Tg, mmol/l	$\bar{x}$	$0.53^{a,b}$	0.49^{c}	$0.45^{d,e}$	$0.78^{b,c,d}$	$1.03^{a,e}$	a	$p < 0.001$
	$\pm$SD	0.09	0.08	0.07	0.18	0.17	b, c, d	$p < 0.05$
							e	$p < 0.01$
U, mmol/l	$\bar{x}$	5.86	5.4	4.88	6.1	5.7	NS	
	$\pm$SD	0.79	0.99	1.58	1.15	1.5		
AST, NE/l	$\bar{x}$	60.2	62.4	33.2	87.8	101.8	NS	
	$\pm$SD	29.8	20.8	15.6	20.9	21.4		

Significant difference (SD $p < 5\%$): The same letters in superscript denote significant differences between the mean values

Blood plasma FFA concentration remained below 0.35 mmol/l, a value considered physiological for ruminants, throughout the experiment. At the 2nd sampling FFA concentration was practically unchanged (0.18 mmol/l) as compared to the control samples. From that time up to the end of the experiment FFA level underwent a significant decrease ($p < 0.05$), finally dropping to one-third of the basic value.

Blood plasma TG concentrations measured at the 2nd and 3rd samplings (0.49 and 0.45 mmol/l, respectively) were not markedly different from the control value (0.528 mmol/l). By the middle of the experimental period, plasma TG concentration increased significantly (0.78 mmol/l, $p < 0.05$), while by the end of the experiment it had doubled (1.03 mmol/l) as compared to the control value.

Blood plasma U concentration measured at different sampling times did not differ significantly from the basic value (5.86 mmol/l).

AST activity was around the upper limit of the physiological range during the experiment. No significant differences were noted at different sampling times.

Analysis of liver biopsy and adipose tissue samples

Total lipid content and fatty acid composition of the liver biopsy samples are shown in Table 4. Mean total lipid content of control liver samples was 56.6 g/kg. This value did not change significantly by the end of fat feeding (67.4 g/kg).

As regards the fatty acid composition of hepatic lipids, the proportion of myristic acid ($C_{14:0}$) and linolenic acid ($C_{18:3}$) significantly decreased ($p < 0.01$ and $p < 0.001$, respectively) by the end of the experiment. At the same time, the proportion of stearic acid ($C_{18:0}$) and oleic acid ($C_{18:1}$) significantly increased ($p < 0.05$ and $p < 0.001$, respectively).

No significant difference occurred in the fatty acid composition of adipose tissue lipids during feeding of the fat-supplemented diet (Table 5).

Volatile fatty acid content of the rumen liquor

VFA concentrations measured in the rumen fluid at different sampling times are shown in Table 6. Only slight fluctuations occurred in total volatile fatty acid (TVFA) concentration and concentrations of individual fatty acids during fat feeding. Significant changes were found only for acetic acid ($C_{2:0}$; $p < 0.05$) and, of the branched-chain fatty acids, isovaleric acid ($C_{5:0}$; $p < 0.1$) concentration rose parallel to the decrease of acetic acid concentration.

Table 4

Total lipid content (g/kg) and fatty acid composition of the liver of the experimental sheep, expressed in per cent of total fatty acids

	Sampling time 1	Sampling time 5	
Total lipid content (g/kg)	58.62 + 17.13 $\bar{x}$ + SD	67.36 + 20.69 $\bar{x} \pm$ SD	
$C_{12:0}$	0.64 ± 0.38	1.00 ± 0.04	$p < 0.01$
$C_{14:0}$	7.82 ± 1.02	2.63 ± 0.99	$p < 0.01$
$C_{14:1}$	1.67 ± 0.04	2.02 ± 0.28	$p < 0.1$
$C_{15:0}$	0.68 ± 0.08	0.74 ± 0.32	NS
$C_{16:0}$	18.79 ± 1.29	19.02 ± 4.51	NS
$C_{16:1}$	1.71 ± 0.77	2.54 ± 0.38	NS
$C_{17:0}$	2.41 ± 0.35	2.34 ± 0.28	NS
$C_{18:0}$	21.27 ± 1.28	23.74 ± 1.57	$p < 0.05$
$C_{18:1}$	20.77 ± 1.72	26.22 ± 3.56	$p < 0.001$
$C_{18:2}$	7.96 ± 1.54	8.03 ± 1.12	NS
$C_{18:3}$	1.06 ± 0.20	0.45 ± 0.18	$p < 0.001$
$C_{20:0}$	0.36 ± 0.11	0.23 ± 0.07	$p < 0.01$
$C_{20:1}$	0.52 ± 0.41	0.40 ± 0.88	$p < 0.05$
$C_{20:3}$	2.09 ± 0.44	0.17 ± 0.11	$p < 0.01$
$C_{20:4}$	5.13 ± 0.79	6.09 ± 2.13	NS
$C_{22:5}$	2.36 ± 0.32	1.13 ± 0.93	$p < 0.01$
$C_{22:6}$	1.91 ± 0.41	2.22 ± 0.26	$p < 0.01$
Other fatty acids	2.87 ± 1.17	1.03 ± 0.13	$p < 0.05$

Table 5

Fatty acid composition of the adipose tissue of experimental sheep, expressed in per cent of total fatty acids

	Sampling time 1 $\bar{x} \pm$SD	Sampling time 5 $\bar{x} \pm$ SD
$C_{12:0}$	0.09 ± 0.03	0.11 ± 0.06
$C_{14:0}$	2.37 ± 0.16	2.39 ± 0.28
$C_{14:1}$	0.61 ± 0.49	0.78 ± 0.79
$C_{15:0}$	0.75 ± 0.20	0.74 ± 0.24
$C_{16:0}$	24.03 ± 1.29	24.66 ± 1.29
$C_{16:1}$	2.26 ± 0.38	2.20 ± 0.41
$C_{17:0}$	2.92 ± 0.66	2.52 ± 0.69
$C_{18:0}$	19.90 ± 4.12	19.07 ± 4.48
$C_{18:1}$	40.96 ± 2.16	40.12 ± 2.75
$C_{18:2}$	4.02 ± 0.87	3.59 ± 0.85
$C_{18:3}$	0.57 ± 0.21	0.39 ± 0.11
$C_{20:0}$	0.60 ± 0.21	0.64 ± 0.25
Other fatty acids	0.90 ± 0.24	0.99 ± 0.66
Saturated/unsaturated fatty acids:	52 : 48	53 : 47

Characteristic changes were noted in the ratios of the three most important volatile fatty acids. Acetic acid/propionic acid and acetic acid/butyric acid ratios decreased during fat feeding.

Table 6

Volatile fatty acid content (mmol/l) of the rumen fluid and ratios of the different volatile fatty acids

Volatile fatty acids (mmol/l)	Sampling time 1	Sampling time 3	Sampling time 5
	$\bar{x} \pm SD$	$\bar{x} \pm SD$	$\bar{x} \pm SD$
Total volatile fatty acids	122.9 ± 28.4	123.4 ± 51.9	122.7 ± 15.7
$C_{2:0}$	$72.6^{a} \pm 19.4$	67.6 ± 30.6	$65.4^{a} \pm 17.7$
$C_{3:0}$	37.5 ± 6.2	37.1 ± 14.5	40.5 ± 8.8
$C_{4:0}$	6.5 ± 2.6	11.9 ± 2.9	7.6 ± 3.0
$iC_{4:0}$	1.7 ± 0.8	1.9 ± 0.4	2.1 ± 0.4
$C_{5:0}$	2.4 ± 1.2	3.0 ± 1.6	2.9 ± 0.9
$iC_{5:0}$	$2.1^{b} \pm 0.9$	2.0 ± 0.9	$4.2^{b} \pm 1.1$
$C_2 : C_3$ ratio	1.9 : 1	1.8 : 1	1.6 : 1
$C_2 : C_3 : C_4$ ratio	62.3 : 32.1 : 5.6	58.0 : 31.8 : 10.2	57.6 : 35.7 : 6.7

SD (p5%): The same letters in superscript denote significant differences between the mean values: a: $p < 0.05$; b: $p < 0.1$

In sacco rumen fermentation experiments

The results of *in sacco* rumen fermentation experiments are summarized in Table 7. Dry matter degradability decreased transiently but nonsignificantly during fat feeding; however, at the last sampling it was identical with that

Table 7

In sacco study of rumen degradability

Sampling time Fat supplementation, %	Dry matter "digestibility" %	Protein "digestibility" %	Crude fibre "digestibility" %
Time 1 Basal ration	79.29 ± 2.93	$^{a}69.55 \pm 11.73$	$^{d}59.08 \pm 4.55$
Time 2 Adaptation phase	77.08 ± 5.55	$^{b}80.72 \pm 24.06$	58.48 ± 24.90
Time 3 Adaptation phase	73.76 ± 4.90	$^{c}75.73 \pm 8.51$	52.45 ± 14.27
Time 4 Experimental feed	74.79 ± 5.48	77.57 ± 8.47	54.30 ± 22.92
Time 5 Experimental feed	77.33 ± 2.20	$^{a,b,c}88.00 \pm 1.54$	$^{d}51.97 \pm 12.12$

Significance (SD, 5%): The same letters in superscript denote significant differences between the mean values: a: $p < 0.01$; b, c, d: $p < 0.05$

obtained for the control sample. No substantial differences were found in the crude protein "digestibility" of alfalfa hay samples at the first four sampling times; at the 5th sampling, however, significantly ($p < 0.05$) elevated values were obtained. On the other hand, in sheep fed the fat-supplemented diet, rumen degradability of crude fibre was poorer both in the adaptation and in the experimental phase than for the baseline samples.

Discussion

In this experiment, 18% of the calculated energy requirement of sheep was met by feeding natural fat of animal origin (fat supplementation constituted 6.6% of the mixed feed). The results were compared to corresponding data obtained for the same animals prior to fat feeding and in the adaptation phase.

At the concentration used in the experiment, fat supplementation caused no disturbances in the health status of ewes, similarly to what was reported by us for dairy cows earlier (Magdus et al., 1988). However, blood plasma variables indicative of lipid metabolism underwent substantial changes.

At the beginning of the experiment all the parameters were in the physiological range.

The most striking changes were found for TL concentration which markedly rose already in the adaptation phase of fat feeding. Astrup et al. (1976) and Palmquist and Conrad (1978) reported similar changes in blood plasma TL concentration for cows fed a fat-supplemented diet. In our experiment, TCh level rose parallel with TL concentration. This is in harmony with the observations of Sharma et al. (1978). The concentrations of both TL and TCh decreased transiently at the 4th sampling; the cause of this decrease is not known. Blood glucose concentration changed in opposite direction. These opposite changes in the concentrations of the three metabolites can probably be attributed to the contrasting effect of hormones (insulin, catecholamines, etc.) which play a role in the regulation of both lipid and carbohydrate metabolism. In our experiment with dairy cows, fat feeding induced no changes in blood plasma insulin concentration (Magdus et al., 1988).

Similarly to the TL level, blood plasma TG concentration increased considerably by the end of the experiment. This lipid fraction did not follow the cyclic changes of TL and TCh: it underwent a continuous increase during fat feeding.

Parallel with the time of feeding fat-supplemented diets, blood plasma FFA concentration decreased continuously. This may be attributed to the fact that fat mobilization from the depots decreased parallel to the ewes' adaptation to fat feeding. The different lipid fractions of the blood plasma underwent

dissimilar changes during fat feeding. Presumably the experimental animals gradually became adapted to fat feeding. Adaptation to fat feeding manifests itself not only in altered fermentative activity of the rumen (Nicholson, 1971) but also in changes of blood plasma composition.

The results indicate that fat supplementation used in this experiment did not constitute a pathological load on the function of hepatocytes. AST activity increased only slightly and nonsignificantly ($p > 0.01$). The TL content of liver biopsy samples remained in the range considered physiological for ruminants.

Liver biopsy samples taken at the end of the experiment showed a significantly ($p < 0.01$) lower ratio of myristic acid, which can be attributed to reduced endogenous synthesis of short-chain fatty acids. Similar changes in milk fat composition in dairy cows have been reported (Storry et al., 1974; Wrenn et al., 1978). The increase in the stearic acid ratio of hepatic lipids either may be due to fat feeding or, theoretically, it may originate from fat mobilization from the depots. Namely, a certain part of the C:18 unsaturated fatty acids of the natural fat fed undergoes hydrogenation in the rumen, thereby increasing the quantity of absorbed stearic acid (Kepler et al., 1966). Three possible explanations may be offered for the rise in oleic acid ratio. The first is that the rumen microflora can saturate only a fragment of the high oleic acid content of dietary crude fat. The second possibility is that polyunsaturated C:18 fatty acids undergo only partial hydrogenation and give rise to oleic acid. The third possible explanation is that, due to the high oleic acid content of the fat depot (40%), already a minor endogenous lipid mobilization may substantially increase the oleic acid content of hepatic lipids. In this experiment, the third possibility was excluded by the fact that no rise in blood plasma FFA concentration, a finding which would point to lipid mobilization, was observed.

Possible changes in deposited fats were studied in the subcutaneous adipose tissue, as in sheep this is the largest fat depot which constitutes about 50% of the mobilizable fat reserve (Wood, 1984). No appreciable changes were found in the fatty acid composition of the fat depots during the experiment. On the other hand, Wrenn et al. (1978) reported that the proportion of myristic acid ($C_{14:0}$) and palmitic acid ($C_{16:0}$) decreased in the adipose tissue of sucking calves given fat-enriched milk. As compared to the fatty acid composition of the dietary fat used in this experiment, the adipose tissue of sheep contained much (1.8, 4.0 and 1.9 times) higher levels of myristic acid, margaric acid and stearic acid. The proportion of oleic acid was nearly the same and that of linoleic acid was less (one-third). In the dietary fat supplement the ratio of saturated/unsaturated fatty acids was 40:60, while in the adipose tissue of ewes it remained practically unaltered during fat feeding (52:48 and 53:47 at the beginning and at the end of the experiment, respectively). The

results suggest that in ruminants the fatty acid composition of dietary fat exerts a less pronounced influence on the fatty acid composition of fat depots than it does in monogastric animals. This phenomenon cannot be explained by the activity of rumen microorganisms alone. Namely, in contrast to the adipose tissue, the fatty acid composition of hepatic lipids underwent substantial changes during fat feeding.

The results of *in sacco* rumen fermentation experiments and the decrease in the acetic acid content of the rumen fluid unambiguously prove that crude fibre digestibility decreased during fat feeding. These results are consistent with the findings of several researchers including Storry and Brumby (1974), Dewendra and Lewis (1974) and Rohr et al. (1978), who reported a reduction in fibre fementation in ruminants fed fat-supplemented diets. As propionic acid and butyric acid concentrations, though not significantly, rose, fat feeding decreased the ratios of acetic acid/propionic acid and acetic acid/butyric acid, a phenomenon observed also by others (Hagemeister and Kaufmann, 1979; Mattos and Palmquist, 1974; Zinn, 1989).

In contrast to fibre fermentation, proteolysis increased during fat feeding, suggesting that the activity of proteolytic microorganisms increased in the rumen content. This must have been accompanied by enhanced activity of protein-synthesizing bacteria, as blood plasma urea concentration, which is considered to be a reliable indicator of energy and protein supply in the rumen, did not change (Oltner and Wiktorsson, 1983).

In summary: from the results it appears that fat feeding exerted a favourable influence on the activity of microorganisms involved in rumen protein metabolism; however, full elucidation and verification of this finding require further experiments.

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FAILURE TO DETECT A PUTATIVE OESTRUS-INDICATING PHEROMONE IN THE URINE OR VAGINAL SECRETIONS OF FEMALE SHEEP

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Extensive use of gas chromatographic analysis of the volatile and non-volatile components of sheep urine and sheep vulvovaginal secretions at different stages of the oestrous cycle has not succeeded in identifying the putative oestrus-indicating pheromone produced by the ewe. However the putative pheromone is probably not a low molecular weight alcohol, diol, phenol, amine, amide, aldehyde, ketone, fatty acid or steroid.

Key words: Pheromone, female, sheep, urine, vaginal secretions, oestrus

Flehmen by the ram is usually observed in a sexual context and in response to urine voided by the ewe. Thus flehmen appears to be involved in the detection of odours (pheromones), in the urine, which indicate the reproductive status of the female (Bland and Jubilan, 1987). The present series of experiments was an attempt to elucidate the nature of this oestrus-indicating pheromone by gas chromatography. The attractiveness of using the gas chromatogram (GC) as the analytical tool is the potential to progress to gas chromatography/mass spectrometry to identify unknown peaks.

General methods

Urine was collected from intact cycling ewes, which were teased daily with a vasectomised ram. Oestrous urine was collected by clipping a small polyethylene bag, with a light plastic jar inset into one corner, over the vulva. The bag was removed immediately after urination occurred in order to avoid any risk of contamination with faeces. On other days of the oestrous cycle the urine was collected manually into a glass or inert plastic container by habituating the ewe to human presence and taking advantage of her reflex urination when approached. Both these methods caused minimal stress to the animal.

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Table 1

Column packings used in the gas chromatograph

Column packing	Separations possible
TENAX-GC	Alcohols, diols, phenols, mono and diamines, amides, aldehydes and ketones
SP1 200	Volatile fatty acids but also a wide range of other organic compounds
CARBOWAX	Volatile fatty acids
OV-17	Wide range of steroids

Vaginal secretions were obtained by swabbing the vulva and vaginal entrance with cotton-wool buds. The buds were then sealed in clean tubes to prevent loss of volatile substances and to avoid contamination. Both urine and vaginal samples were either stored at −10 °C or used immediately. Both states were used in most of the experiments.

Analysis of all samples was by gas-liquid chromatography as this would allow eventual identification of any pheromonal compound found by mass spectroscopy. The machine used was a Hewlett-Packard 5700A gas chromatograph, fitted with a flame-ionisation detector, and a Hewlett-Packard 7123B chart recorder. In some instances an integrator was interposed in the circuit. The mammalian pheromones that have already been indentified belong to a wide range of organic compounds. Thus four different column packings have been used in the gas chromatograph (GC) to try to embrace as wide a range of potential compounds as possible (see Table 1). Details of extraction methods and temperature conditions are given under the different experiments. A variety of statistical tests were used.

Procedures and results

Experiment 1: Analysis of the volatile components in sheep urine

This experimental procedure was based on the method of Zlatkis et al. (1973). The urine sample was heated up to release the volatile components which were then trapped in a sampling tube. The sampling tube containing the volatiles was then purged to transfer these volatiles into the GC for analysis.

Method. The sampling tube consisted of a 7″ stainless steel tube (I. D. 1/4″) packed with TENAX-GC 35/60 mesh (Applied Science Laboratories Inc., Pennsylvania). Five ml aliquots of fresh or frozen urine were heated in a round-bottomed flask over a boiling water-bath. The sampling tube was connected to

the flask via a reflux condenser (to prevent water vapour entering the sampling tube) and the urine refluxed for 1 h. A continual stream of carrier gas (N_2, 10 ml/min) passed over the heated urine and out through the sampling tube.

The sampling tube was then quickly connected to the GC injection port. A constant flow of carrier gas (N_2, 20 ml/min) was maintained through the tube while it was purged for 10 min at 200–210 °C by means of an insulated resistance-wire jacket. This drove the volatiles into a 2′ glass column (I. D. 1/8″), packed with TENAX—GC, in the GC oven at room temperature. After allowing the sampling tube to cool but without interrupting carrier gas flow, the GC oven programme (30 °C for 3 min, then 8 °C/min to 200 °C) was started. The flame-ionisation detector temperature was 250 °C.

Results. This method showed good reproducibility on aliquots of the same sample. Also fresh urine and frozen samples of the same urine gave very similar chromatograms (Fig. 1). A total of 64 different urine samples from 4 normally cycling ewes were analyzed and the peak areas resulting measured and compared (Fig. 2). Some 10 to 20 peaks were present in the chromatograms and 9 of these peaks occurred in all samples. Exhaustive statistical analysis failed to reveal any peak consistently absent or less at oestrus than at other stages of the cycle or vice versa.

This inability to identify a specific peak that could be a candidate for the putative oestrus-indicating pheromone could be due to a number of factors:

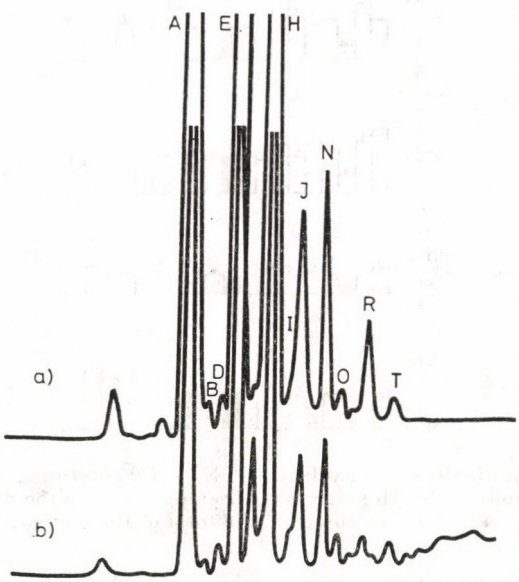

Fig. 1. Gas chromatograms showing the peaks representing the volatile compounds released from 5 ml of ewe urine. *a)* urine sample initially frozen; *b)* fresh urine sample. The peaks are identified by letters to ease comparison with Fig. 2

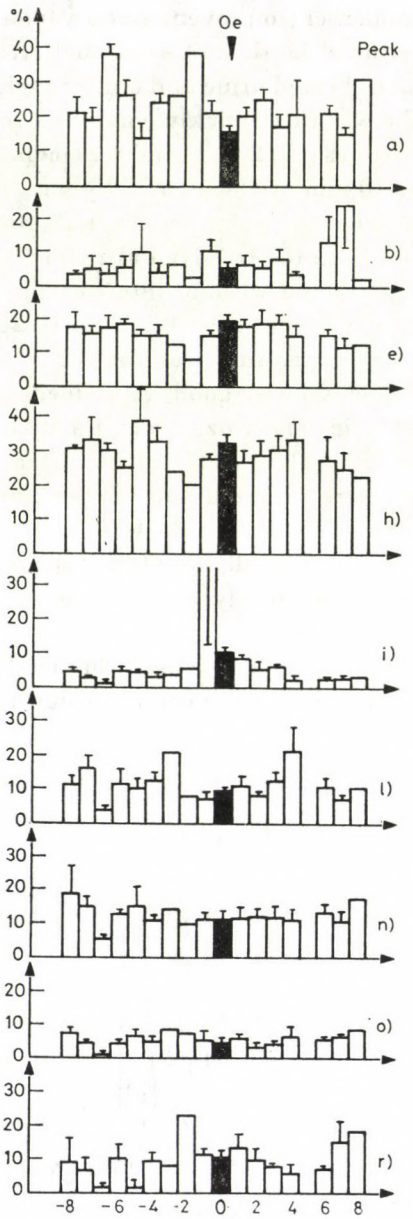

Fig. 2. Histograms showing the mean values (± SEM) of 9 constituents of ewe urine on different days of the oestrous cycle. All values have been pooled from the different ewes (n = 64). The values are expressed as percentage of the total of these constituents for each day

(a) Amount of putative pheromone present below detection level of GC; (b) GC column not suitable for chemical nature of putative pheromone; (c) Putative pheromone not volatile.

Behavioural observations by this laboratory (Bland and Jubilan, 1987) have shown that flehmen response by the ram was of greater duration to urine than to vaginal secretions. This was taken to indicate that the putative pheromone must be more concentrated in the latter location than in the urine; the premise being that the more concentrated the odour the less time needed to activate the sensory reseptors. The analysis of vaginal secretions would thus improve chances of detection and the use of a wider range of column packings would further increase this.

Experiment 2: Analysis of the volatile components of sheep vaginal secretions

Three procedures using a similar principle to Experiment 1 were used.

(a) Vaginal swabs were heated over a boiling waterbath such that the volatile components were carried into a metal sampling tube packed with TENAX-GC by a stream of carrier gas (He, 10 ml-min). The sampling tube was then attached to the injection port of the GC and treated as in Experiment 1.

(b) Vaginal swabs were heated over a boiling water-bath such that the volatile components were carried into a 6″ glass precolumn (I. D. 1/4″) packed with 10% SP-1200 with 1% H_3PO_4 on 80/ 100 Chromosorb W (carrier gas: He, 10 ml/min). The precolumn was then inserted between the injection port and a 6′ steel column (I. D. 1/8″) packed with SP-1200. The oven was temperature programmed for 2 min at 40 °C, then 2 °C/min up to 150 °C. The flame-ionisation detector was set at 200 °C.

(c) Vaginal swabs were heated over a boiling water-bath such that the volatile components in the carrier gas (He, 10 ml/min) passed into a S-shaped "fermentation trap" containing 5 ml acetone or ethyl acetate. The volatiles dissolved in the solvent in the "trap" and 1 μl aliquots of this solvent were then injected onto a 2′ glass column (I. D. 1/8″) packed with 3% OV-17 on 80/90 Diatomite CLQ and running isothermally at 275 °C. The injector and detector temperatures were 300 °C.

Results. The method using TENAX columns gave fair separation with 7 to 10 peaks making up the chromatogram; of these peaks 6 occurred in all 25 samples processed. SP-1200 gave the best separation of volatile components producing 15–24 peaks per chromatogram although only 8 of these appeared to be common to all the 58 samples analyzed. The use of the OV-17 columns by this method was not successful, yielding only a single peak on the print-out. It was thus abandoned after only 8 samples. Again no peak (or peaks) could be found which was consistently present at oestrus and absent or reduced at other stages of the cycle or vice versa.

A recent work has suggested that pheromonal substances do not need to be very volatile (Wysocki et al., 1985). Direct extraction of urine or preferably vaginal secretions with solvents would be needed to reveal such non-volatile components.

Experiment 3: Analysis of the non-volatile components of sheep urine

Methods. (a) Two ml urine was acidified with 2N H_2SO_4 until pH between 2 and 3 and then extracted with 6 ml diethyl ether. Two μl extract injected onto 10′ metal column (I. D. 1/8″) packed with 3.5% CARBOWAX 4000 monostearate with 0.35% H_3PO_4 Chromosorb G 70/80 mesh (carrier gas: He, 30 ml/min; injector: 150 °C; oven isothermal: 125 °C; detector: 200 °C).

(b) Two μl urine injected directly into 2′ metal column (I. D. 1/8″) packed with TENAX-GC. Oven programmed for 2 min at 40 °C then 8 °C/min to 250 °C (carrier gas: N_2, 10 ml/min; injector and detector: 270 °C). This method was also used with dual column compensation.

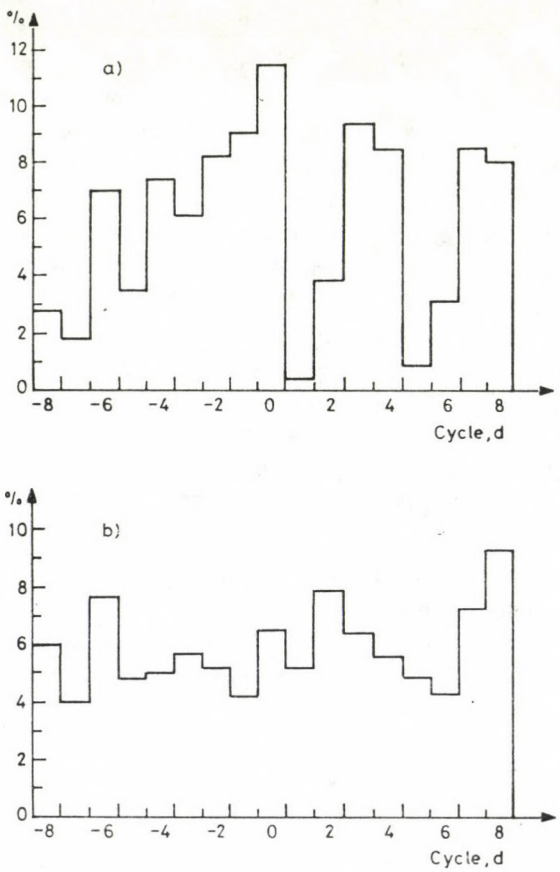

Fig. 3. a) Histogram showing the total amount of the 6 "fatty acids" from 1 ml sheep urine, for each day of the cycle expressed as a percentage of the total "fatty acid" concentration for the whole cycle. *b)* Histogram showing the total amount of the 6 "fatty acids" from a single ovine vaginal swab, for each day of the cycle expressed as a percentage of the total "fatty acid" concentration for the whole cycle

(c) Four parts urine were extracted with one part hexane; the hexane was then concentrated 40 times and 1 μl injected onto a 6′ glass column (I. D. 1/8″) packed with 3% OV-17 on 80/90 Diatomite GLQ (carrier gas: N_2, 60 ml/min; injector: 300 °C; oven isothermal: 230 °C; detector: 300 °C).

Results. The running of acidified urine extracts on CARBOWAX revealed that most of the 20 urine samples processed contained substances that had the same retention times as acetic, propionic, isobutyric, butyric, isovaleric and valeric acids. However none of these individual "fatty acid" concentrations showed a significant relationship with the oestrous cycle. The number of "fatty acids" varied between the different days and so did the relative proportions of each. The total amount of all the "fatty acids" for a particular day increased gradually from 8 days before oestrus to peak at oestrus ($r = 0.8868$; $P < 0.05$; Fig. 3). However when all values from all the days of the cycle were considered, the relationship with the oestrous cycle was not significant ($r = 0.4314$). None of the unidentified peaks showed a correlation with the oestrous cycle.

Direct injection onto TENAX gave chromatograms with from 15 to 30 peaks each. Even using dual column compensation reproducibility was poor and a good baseline was very difficult to obtain. However some 60 chromatograms from 8 urine samples (either oestrus or day 9) failed to highlight any peak that could be associated with the putative oestrus-indicating pheromone.

The results of the hexane-extracted urine will be discussed after Experiment 4.

Experiment 4: Analysis of the non-volatile components of sheep vaginal secretions

The aim in this experiment was to look at the supposedly most concentrated source of the pheromone by direct extraction.

Methods. (a) Vaginal swabs were acidified with 2N H_2SO_4 and extracted with 25 ml diethyl ether. The extract was concentrated by extracting the ether with 2 ml N NaOH; after reacidifying with 2N H_2SO_4 to pH 2–3, the soda was extracted with 6 ml diethyl ether. One μl of this final ether extract was introduced onto a 10′ metal column (I. D. 1/8″) packed with 3.5% CARBOWAX 4000 monostearate with 0.35% H_3PO_4 on Chromosorb G, 70/80 mesh (carrier gas: He, 30 ml/min; injector: 150 °C; oven isothermal at 125 °C; detector: 200 °C). Simple extracts of vaginal swabs with methanol were also tried with this column but they gave poor results.

(b) Single vaginal swabs were extracted with 1 ml hexane which was then concentrated four-fold. One μl of this extract was injected onto a 6′ glass column (I. D. 1/8″) packed with 3% OV-17 on Diatomite CLQ 80/90 mesh (carrier gas: N_2. 80 ml/min; injector and detector: 300 °C; oven isothermal at 230 °C).

Results. As with the acidified extracts of urine the 20 extracts of vaginal swabs run on GARBOWAX 4000 all contained peaks with similar retention times to the low molecular weight volatile fatty acids. The total "fatty acid" concentration of vaginal swabs varied little from day to day, also no individual "fatty acid" or unknown peak showed consistent correlation with the stage of the oestrous cycle.

The results of hexane-extracted urine and vaginal swabs run on OV-17 were analyzed together on the premise that only peaks occurring in the chromatograms of both urine and vaginal samples were likely candidates for the pheromone.

Although some 11 peaks were present in the extracts of vaginal swabs and 8 in extracts of urine and several of these peaks were common to extracts from both sources, none of these common peaks occurred only in oestrous samples, as opposed to non-oestrous samples, or vice versa. One of the common peaks had the same retention time as the steroid, 5α-androst-16-ene-3-one, one of the active ingredients of the boar pheromone that potentiates oestrous behaviour in the sow (Melrose et al., 1971), but the size of this peak did not vary consistently with the stage of the cycle.

Discussion

In spite of the incompleteness of the scope of the present study and the disappointing results obtained, it has effectively eliminated approximately 20–30 volatile and a similar number of non-volatile components of urine and vulvovaginal secretions as candidates for the putative oestrus-indicating pheromone. The only positive correlation obtained was the increasing total volatile fatty acid content of urine in the week prior to oestrus, but the absence of a similar trend in vaginal secretions makes the correlation unlikely to be related to the putative pheromone. The high concentrations of volatile fatty acids in the eructated gases of sheep also mitigates against this probability. However it is conceivable that flehmen could be involved in the removal of volatile fatty acids from the nasal cavity so that these compounds can be monitored without high indigenous background levels. There is currently no evidence in support of this, although flehmen is very pronounced in ruminants as compared to other groups with the exception of the Equidae – the non-patency of the nasopalatine duct may account for its exaggerated nature in this latter group. The present experiments suggest that the putative oestrus-indicating pheromone in sheep is probably not a volatile or non-volatile low molecular weight alcohol, diol, phenol, amine, amide, aldehyde, ketone, fatty acid or steroid. The present methods unfortunately do not allow firm conclusions unless a positive correlation of one or more compounds with the stage of

the oestrous cycle is obtained. In order to optimize results further use of the present approach by varying the type of column packing should be based on Experiment 3(c) versus Experiment 4(b). Future work also needs to concentrate on the development of a bioassay, which will allow partial purification of the putative pheromone prior to chemical analysis. Such an assay cannot be based on flehmen behaviour as most of the available evidence now indicates that flehmen may only be involved in getting the pheromone to the receptors where it can be assessed (Bland and Jubilan, 1987). Thus flehmen occurs before pheromone assessment and not as a result of it.

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IMMUNOREACTIVE PROLACTIN, PROGESTERONE AND LUTEINIZING HORMONE IN THE SEMINAL PLASMA OF BUFFALO *(BUBALUS BUBALIS)*

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The presence of immunoreactive prolactin, luteinizing hormone (LH) and progesterone in buffalo seminal plasma is reported for the first time. Correlations were obtained between various semen attributes and the levels of the above immunoreactive hormones. Statistically significant ($P < 0.01$) negative correlations were found between the levels of immunoreactive prolactin in semen and sperm motility and viability. The coefficient of multiple linear correlation (R^2) between the levels of immunoreactive progesterone, prolactin and LH in the seminal plasma and the various attributes of semen revealed that immunoreactive progesterone and prolactin showed stronger interactions than did LH. The biological significance of these immunoreactive hormones in semen is discussed.

Key words: Prolactin, progesterone, luteinizing hormone, semen attributes

Seminal plasma is a highly complex biological fluid that constitutes the vast majority of buffalo semen. Its physiological functions, however, are not very well understood (Sidhu and Guraya, 1985, 1989). Although a wide variety of biomolecules such as enzymatic and nonenzymatic proteins, lipid derivatives, ions, sugars, adenine nucleotic, motility and decapacitation factors have been reported, we do not know much about their effect on the fertility of semen (Seigal et al., 1987). Interestingly, hormones such as prolactin, insulin, testosterone, dihydrotestosterone, luteinizing hormone (LH), follicle stimulating hormone (FSH) and prostaglandins have also been reported to occur in the semen of some mammalian species (Abbaticchio and Giorgino, 1983; Hyne and Boettcher, 1977; Iosefi and Lewin, 1984; Sheth et al., 1975, 1976, 1977; Sluss et al., 1987; Sueldo et al., 1985). Some preliminary attempts have been made to correlate the level of these hormones with the quality and fertility of semen (Sheth et al., 1977; Sueldo et al., 1985). In buffalo, studies of the impact of some steroid and protein hormones on the physiology of spermatozoa have been reported (Sidhu and Guraya, 1985), and these hormones are also exploited for effective preservation of the semen (Sidhu and Guraya, 1979*b*; 1985). There is no report in the literature about the levels of various hormones in buffalo semen and the relationships of these hormones with the quality of semen. This fact prompted us to carry out the present investigation. The presence of prolactin, LH and progesterone in buffalo semen is reported here for the first time.

Materials and methods

Semen collection. Semen samples were collected using artificial vagina from each of four 5- to 6- year old bulls twice a week. The samples were maintained at 37 °C in the laboratory. All semen samples were analyzed within 30 min of collection for volume, pH, sperm concentration, mass motility, percentage motility, percentage viability and percentage of sperm abnormality according to the procedures described by Sidhu and Guraya (1985).

Separation of seminal plasma from the semen. The ejaculated semen samples were layered on 1 M sucrose containing 0.02% sodium azide and 50 mM benzamidine, and were centrifuged at 2000 *g* for 30 min at room temperature. The supernatant seminal plasma was collected using Pasteur pipettes and preserved at —20 °C until used for hormone determinations. Care was taken to avoid contamination of the seminal plasma with the cytoplasmic droplets which are usually collected at the seminal plasma—sucrose interface after centrifugation.

Determination of hormones by radioimmunoassay (RIA). Prolactin, LH and progesterone were determined in 50 μl aliquots of the seminal plasma directly, without extraction, using commercial RIA kits obtained from the Diagnostic Products Corporation (U. S. A.) and Bhaba Atomic Research Centre, Trombay, Bombay (India). Progesterone was assayed using antibody-coated tubes. Standard curves were prepared for each hormone.

Statistical methods. The relationship between hormones and various semen characteristics was derived by calculating coefficients of correlation (r). In order to find out how much of the variation in hormone levels contributed to variations in the semen parameters, a coefficient of multiple linear correlation (R^2) was calculated between the levels of hormones (x), semen volume (x_1), pH (x_2), motility (x_3), viability (x_4), abnormal forms (x_5) and sperm concentration (x_6). The data obtained for samples were computed using the following equation:

$$x = a + b_1x_1 + b_2x_2 + b_3x_3 + b_4x_4 + b_5x_5 + b_6x_6.$$

Results and discussion

Immunoreactive progesterone, LH and prolactin were determined by radioimmunoassay in semen samples taken from 24 buffalo bulls. The levels of these hormones were calculated from standard curves prepared simultaneously. The mean values of immunoreactive progesterone, prolactin and LH, and various characteristics of buffalo bull semen are given in Table 1. These hormones were present predominantly in the seminal plasma but were also detected (data not shown) in sperm homogenates, indicating the binding of these hormones to sperm surface. The levels of immunoreactive progesterone and prolac-

tin hormones in buffalo seminal plasma are within the normal range reported for the serum, whereas those of LH are of higher range.

The presence of various immunoreactive hormones, i.e. prolactin, FSH, LH, insulin, testosterone, 17ß-oestradiol etc. has been documented in the seminal plasma of humans, bulls and boars (Sheth et al., 1976, 1977; Abbaticchio and Giorgino, 1983; Iosefi and Lewin, 1984; Sueldo et al., 1985; Sairam et al., 1980; Ramasharma et al., 1986; Fecchinetti et al., 1987). The presence of immunoreactive progesterone, LH and prolactin in buffalo bull semen is reported here for the first time.

Table 1

Levels of immunoreactive progesterone, prolactin and LH and various parameters of buffalo bull semen

Parameters	Levels	
	*Mean ± S.E.	Range
A. Hormones		
Progesterone (ng/ml)	0.51 ± 0.39	0.21– 1.50
Prolactin (ng/ml)	39.23 ± 20.26	30.0– 100.0
LH (mIU/ml)	51.53 ± 49.90	0.0– 130.0
B. Semen parameters		
Volume (ml)	3.11 ± 0.53	2.0– 4.3
pH	6.71 ± 0.43	6.0– 7.5
Motility (%)	52.12 ± 2.40	10.0– 80.0
Viability (%)	62.53 ± 1.72	12.0– 85.0
Sperm abnormality (%)	15.68 ± 4.42	8.0– 25.0
Sperm concentration (x 10^7/ml)	83.25 ± 13.13	70.0– 111.0

* Means of 24 semen samples from four buffalo bulls

Table 2

Correlation coefficients (r) between the levels of progesterone, prolactin and LH and various parameters of buffalo bull semen

Semen parameters	Correlation coefficients (r)		
	Progesterone	Prolactin	LH
Volume	0.30	0.06	–0.01
pH	–0.17	0.05	–0.35
Motility	–0.35	–0.52	–0.03
Viability	–0.41	–0.052	–0.06
Abnormals forms	0.31	0.18	0.09
Concentration	–0.06	0.24	0.25

In the present study, attempts were also made to correlate the levels of immunoreactive progesterone, prolactin and LH with various characteristics of buffalo bull semen such as volume, pH, percentage of abnormal forms, and sperm concentration (Table 2).

Immunoreactive LH was in a negative correlation with all the parameters except abnormal forms and sperm concentration as also reported for bulls by Razdan et al. (1976). However, a positive correlation between semen LH levels and sperm counts has been reported by other workers (Sheth et al., 1975, 1976; Biswas et al., 1978). Immunoreactive prolactin showed a negative correlation with sperm motility and viability. In contrast, Sheth et al. (1975) observed higher prolactin levels in human semen samples with a sperm motility higher than 30%. However, Sueldo et al. (10) reported that men having high prolactin levels in the semen showed lower sperm motility as was also observed by Seigal et al. (1987). Positive correlations found between immunoreactive prolactin and semen volume, semen pH, percentage of abnormal sperm and sperm concentration are similar to those reported for bull semen by others (Razdan et al., 1976; Biswas et al., 1978). Prolactin is considered to be a "stress hormone" and various stressors (e.g. adverse seasonal effects) trigger its release which, in turn, might influence the physiology of the accessory reproductive glands. Prolactin has physiological significance as it increases cAMP levels, fructose utilization, glucose oxidation and ATPase activity, decreases the tetracycline binding of human spermatozoa and also alters the ionic state of spermatozoa.

Immunoreactive progesterone showed a positive correlation between semen volume and abnormal forms of sperm, and negative correlations between semen pH, sperm motility, sperm viability and sperm count (Table 2). Its biological significance is obscure. Amann and Hammerstedt (1976) showed that progesterone binds to intact bovine spermatozoa with great affinity, followed by estradiol, dihydrotestosterone, androsterone and androstenediol. Warikoo et al. (1986) proposed that the distribution of testosterone binding sites on monkey sperm determined the motility and fertility of semen. Sidhu and Guraya (1980) showed that buffalo spermatozoa have a capacity for steroid interconversions that may be important for creating an androgenic environment for spermatozoa, as androgens have been shown to affect sperm and metabolism (Beck et al., 1976; Sidhu and Guraya, 1979*a*, 1985).

The coefficients of multiple linear correlation (R^2) between the levels of various hormones and semen characteristics (semen volume, pH, per cent motility, per cent viability, per cent abnormality and sperm concentration) also calculated in this study revealed an interaction between the levels of hormones and various semen characteristics. Immunoreactive progesterone and prolactin showed stronger interactions than did immunoreactive LH. More than 32% of the variation in the levels of progesterone and more than 35% of the variation in prolactin levels could be explained by, or were due to, variations in these

semen characteristics, whereas only 23% of the variation in the levels of immunoreactive LH was accounted for by the variations in these semen characteristics.

The presence of immunoreactive progesterone, prolectin and LH in buffalo bull semen and their relationships with various semen attributes propose the question of their biological significance and mode of action, if any, in spermatozoa. With the exception of triiodothyronine and calmodulin, no other protein hormones elicit any physiological response in mammalian sperm (Sidhu and Guraya, 1985, 1989). Similarly, Amann and Hammerstedt (1976) showed that only few saturable, high-affinity steroid binding sites exist in bull spermatozoa. We propose that these hormones nonspecifically interact with the sperm plasma membrane and change its permeability to various metabolic substrates, as FSH, LH, prolactin and testosterone have been shown to affect the metabolism of buffalo sperm (Sidhu and Guraya, 1979*a;* Bhela et al., 1981).

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EFFECT OF DIETARY AMINO ACIDS ON *IN VITRO* RUMEN BACTERIAL PROTEIN SYNTHESIS IN BUFFALOES

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The effect of different ratios of urea to amino acid N at a fixed concentration of soluble sugars as energy source and varying levels of soluble sugars at optimum urea cell suspension was obtained from the rumen fluid of buffalo *(Bubalus bubalis)* calves fed on a growth ration. Under glucose fermentation, the bacterial protein content of the incubation mixture (I. M.) was increased to 3.91, 6.31 and 5.08 times the control value (urea alone) when 25, 50 and 75% of urea-N was replaced with amino acid N, respectively. With cellobiose, the corresponding increase was 4.06, 5.29 and 5.63 times. At 50% urea-N replacement with amino acid N (a ratio for maximum protein synthesis), the bacterial content was maximum when 1 g glucose or cellobiose per 100 ml of I. M. was added. Per cent incorporation of radioactivity from amino acids into bacterial protein was maximum at 25% amino acid N level with both the soluble sugar sources. The total amino acids incorporated into bacterial protein were, however, more at 50% than at 25% amino acid N level.

Key words: Dietary amino acids, urea, protein synthesis, rumen bacteria, ruminants

Dietary proteins are catabolized to peptides and amino acids in the rumen and most of the amino acids pass through the ammonia pool prior to incorporation into microbial protein (Wright and Hungate, 1967*a*, *b*). However, during this process all the ammonia produced, especially during the first hours after feeding, is not utilized for microbial protein synthesis (Chalupa, 1975) and the excess is lost as urea in the urine (Nolan and Leng, 1972). Maximizing the direct incorporation of amino acids of dietary origin into microbial protein in the rumen, therefore, would be an important approach to increasing the efficiency of utilization of dietary proteins. This *in vitro* study deals with the effect of different ratios of urea to amino acid N at a fixed concentration of soluble sugars and, conversely, of varying levels of soluble sugars at a fixed urea to amino acid N ratio on *in vitro* rumen bacterial protein synthesis and related parameters.

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Materials and methods

The procedure used for the withdrawal of rumen fluid, the buffalo *(Bubalus bubalis)* calves and their feeding were the same as described earlier (Sood et al., 1991). Bacteria were separated from the rumen fluid by the procedure and using the rumen buffer solution (RBS) described by Maeng et al. (1976). The washed bacterial cells were suspended in a volume of CO_2-saturated RBS, maintained at 39 °C, equivalent to one-half of the original volume of rumen fluid.

Experiment 1. For studying the effect of different ratios of urea to amino acid N (AAN) on various rumen parameters, each fermentation flask fitted with a stopper and bunsen valve (Johnson, 1966) contained, in 20 ml final volume of CO_2-saturated PBS, 200 mg glucose or cellobiose, 23.4 mg urea (10.8 mg N) or 17.4 mg urea (8.1 mg N) + 17 mg mixed amino acids (18 amino acids mixed on equimolar basis) (2.7 mg N) or 11.6 mg urea (5.4 mg N) + 34 mg mixed amino acids (5.4 mg N) or 5.8 mg urea (2.7 mg N) + 51 mg mixed amino acids (8.1 mg N) and 6.8 ml washed bacterial cell suspension. The flasks were incubated at 39 °C in an atmosphere of CO_2 for 20 min before the addition of washed bacterial cell suspension and then for 2, 4, 6 and 8 h for ammonia and total volatile fatty acids (TVFA) estimations and for 10 h for measurement of bacterial protein synthesis in a temperature-controlled water bath with shaking platform. Zero-hour controls were run simultaneously. At the end of the incubation period, the fermentation was stopped by adding 1 ml of saturated mercuric chloride solution to each flask. 10.8 mg urea-N (UN), 8.1 mg UN + 2.7 mg AAN, 5.4 mg UN + 5.4 mg AAN and 2.7 mg UN + 8.1 mg AAN were designated as 0, 25, 50 and 75% AAN levels, respectively.

Experiment 2. For studying the effect of different concentrations of glucose or cellobiose at fixed urea to AAN ratio on bacterial protein synthesis, each flask contained, in 20 ml final volume of CO_2-saturated RBS, 5.4 mg UN + + 5.4 mg AAN, 50, 100, 150, 200 or 250 mg of either glucose or cellobiose and 6.8 ml washed bacterial cell suspension. The flasks were incubated as in experiment 1 for 10 h.

Experiment 3. Experiment 1 was repeated but 4, 8 and 12 μCi of chlorella protein hydrolysate $-C^{14}$ (U) was also added to flasks containing 25, 50 and 75% AAN, respectively, to examine the effect of different ratios of urea to AAN on the incorporation of radioactivity from amino acids into bacterial protein.

Ammonia-N and TVFA in the incubation mixture (IM) was determined by the procedure described by Conway (1950) and Kromann et al. (1967), respectively. Proteins were precipitated from the IM with ethanol (McLaren et al., 1976), dissolved in 0.1N NaOH by heating at 70 °C for 30 min, the cell debris was removed by centrifugation and the protein content of the super-

natant was estimated by the Lowry method (Lowry et al., 1951). Radioactivity was measured in a liquid scintillation spectrometer using the scintillation fluid described by Bray (1960).

Results

Experiment 1. AAN substitution at various levels for UN markedly increased bacterial protein content in the IM (Table 1). With glucose as the source of energy, the net bacterial protein content was 9.69, 37.85, 61.10 and 49.18 mg/100 ml IM when 0,25, 50 and 75% of UN was replaced with AAN, respectively. The corresponding values with cellobiose were 9.48, 38.49, 50.16 and 53.39. Bacterial protein content was, therefore, maximum at 50% and 50 and 75% AAN when glucose and cellobiose was the source of energy, respectively.

Higher accumulation of ammonia occurred in the IM when up to 50% of UN was replaced with AAN at almost all the incubation times and under both

Table 1

Effect of different urea to amino acid N (AAN) ratios upon rumen bacterial protein synthesis during glucose or cellobiose fermentation (n = 4)

Incubation time (h)	AAN, %	Bacterial protein (mg/100 ml IM) during fermentation of	
		Glucose	Cellobiose
0		11.88	11.88
10	0	21.57	21.36
	25	49.73	50.37
	50	72.98	62.04
	75	61.06	65.27

Table 2

Effect of different urea to amino acid N (AAN) ratios on the content of ammonia-N during glucose or cellobiose fermentation (n = 2)

Substrate	Incubation (h)	Ammonia-N (mg/100 ml IM)			
		0% AAN	25% AAN	50% AAN	75% AAN
Glucose	2	2.21	3.71	4.55	3.71
	4	7.18	8.51	7.32	5.81
	6	6.90	9.21	8.30	6.34
	8	5.36	5.01	5.43	4.34
Cellobiose	2	3.74	3.97	2.88	2.49
	4	6.80	9.60	6.95	3.67
	6	7.90	11.03	9.79	6.31
	8	6.88	13.53	8.54	6.01

glucose and cellobiose fermentation as compared to when urea alone was the source of nitrogen (Table 2). Accumulation of ammonia was maximum at 25% AAN. Under glucose or cellobiose fermentation, the total volatile fatty acid (TVFA) content of the IM was decreased at all the levels of AAN substitution and incubation times (Table 3). The decrease was parallel with increasing levels of AAN.

Experiment 2. At 50% AAN (optimum urea to AAN ratio for bacterial protein synthesis, Table 1), the bacterial content in the IM was maximum when 1 g/100 ml IM of glucose or cellobiose was added (Table 4). Increasing the concentration of glucose or cellobiose from 0.25 to 1.0 g/100 ml IM proportionately increased the bacterial protein content.

Experiment 3. Per cent incorporation of radioactivity from labelled amino acids into bacterial protein was maximum at 25% AAN level under both glucose and cellobiose fermentation (Table 5). Total amino acids incorporated

Table 3

Effect of different urea to amino acid N (AAN) ratios on the content of total volatile fatty acids during glucose or cellobiose fermentation (n = 2)

Substrates	Incubation time (h)	Total volatile fatty acids (mmol/100 ml IM)			
		0.1 AAN	25% AAN	50% AAN	75% AAN
Glucose	2	5.85	4.35	4.65	4.40
	4	6.00	5.14	4.47	4.30
	6	5.39	4.95	4.25	4.20
	8	4.10	3.25	2.25	1.95
Cellobiose	2	5.37	5.02	5.16	5.09
	4	5.79	4.81	4.88	3.56
	6	6.28	5.58	4.12	3.91
	8	5.86	5.60	4.53	3.28

Table 4

Effect of different concentrations of glucose or cellobiose on rumen bacterial protein synthesis at a fixed urea to amino acid nitrogen (AAN) ratio (50% AAN; n = 4)

Incubation time (h)	Glucose concentration used (g/100 ml IM)	Bacterial protein (mg/100 ml IM)	Cellobiose concentration (g/100 ml IM)	Bacterial protein (mg/100 ml IM)
0		12.13		12.13
10	0.25	40.92	0.25	41.44
	0.50	53.53	0.50	49.67
	0.75	66.91	0.75	58.16
	1.00	88.04	1.00	66.91
	1.25	72.32	1.25	56.88

Table 5

Effect of different urea to amino acid N (AAN) ratios on the incorporation of radioactivity from ^{14}C-labelled amino acids into rumen bacterial protein during glucose or cellobiose fermentation

Incubation time (h)	AAN %	During glucose fermentation		During cellobiose fermentation	
		Total amino acids incorporated into bacterial protein (mg)	Radioactivity incorporated into bacterial protein (%)	Total amino acids incorporated into bacterial protein (mg)	Radioactivity incorporated into bacterial (%)
10	25	23.11	27.19	22.21	26.13
	50	44.27	26.04	29.53	17.37
	75	40.49	15.88	35.79	14.03

into bacterial protein increased as the amount of AAN in the IM was increased up to 50% and 75% in the case of glucose and cellobiose, respectively.

From the results of these experiments it can be inferred that maximum rumen bacterial protein synthesis occurred when 50% of the total nitrogen was supplied in the form of amino acid in the IM. However, considering the cost factor, at least 25% of the total nitrogen should be supplied as amino acid/protein as per cent incorporation of amino acids into bacterial protein was maximum and protein synthesis was also considerable at this level.

Discussion

It has been suggested that NPN to amino acid ratio may be an important factor influencing rumen microbial protein synthesis. The results of the present study, i.e. that more bacterial protein synthesis occurred when urea-N was replaced with various levels of amino acid N (Table 1), confirmed the above suggestion and supported the summary remarks by Morris et al. (1975) that diets with a high proportion of nitrogen as NPN do not support rumen microbial growth rates or efficiencies of energy utilization for microbial synthesis due to the lack of the required amount of amino acids. The higher accumulation of ammonia in the IM with AAN substitution, particularly at 25% AAN level, as compared to urea alone (Table 2) also indicated preferential uptake of amino acids to ammonia for increased protein synthesis by rumen bacteria. The decreased concentration of ammonia in the IM containing 50 and 75% as compared to that containing 25% AAN, however, may be due to the lower amount of urea added. The radiotracer experiment (Table 5) further confirmed the possibility that the increase in bacterial protein synthesis with amino acids substitution for urea was due to the incorporation of added amino acids into bacterial protein. The increased bacterial protein synthesis obtained with higher levels of glucose or cellobiose addition (Table 4) showed that the amount of

carbohydrates can influence the uptake of amino acids by rumen microorganisms to be incorporated into their body proteins. Similar were the views of Stern et al. (1978), Baldwin and Allison (1983) and Windschitl and Schingoethe (1984).

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COMPARATIVE STUDIES ON *IN VITRO* MITOGEN-INDUCED PROLIFERATION OF PERIPHERAL BLOOD LYMPHOCYTES IN DOG AND BREEDING FOX

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In vitro blastogenesis of dog and fox lymphocytes was compared by a microculture technique. The highest ^{3}H-thymidine incorporation in cultures of dog lymphocytes was observed at day 3, while in those of fox at day 2, incubated either at 37 °C or at 39 °C. Lymphocytes cultured at 39 °C incorporated more tritiated thymidine than did cells cultured at 37 °C. The stimulation index (SI) of dog peripheral blood lymphocytes to both mitogens concanavalin A (Con A) and leucoagglutinin (LA) was in a similar range, while pokeweed mitogen (PWM) showed a weaker but significant stimulatory action. The blastogenesis of fox lymphocytes was the greatest in Con A stimulated cultures. The mitogenic potency of LA and PWM was about half of that of Con A, with no essential difference between them. Maximum lymphocyte proliferation of dog and fox was observed when culture media were supplemented with 10% fetal calf serum (FCS).

Key words: Dog, fox, lymphocyte, stimulation test, mitogens

Lymphocte reponse to mitogens is a method widely applied for estimating cellular immune function in various species of domestic and laboratory animals (Barta, 1984; Kok-Mun Tham et al., 1982; Muscoplat et al., 1974; Kristensen et al., 1982). One of the best known techniques involves incorporation of tritiated thymidine (^{3}H-Tdr), a radioactively labelled DNA precursor. However, the amount of ^{3}H–Tdr incorporated in cultured lymphocytes *in vitro* depends on many factors including biological, e.g. variation in individual reactivity, circannual rhythm, age of blood donors (Hume and Weidemann, 1980; Ling and Kay, 1974; Angus and Yang, 1978; Gerber and Brown, 1974), chemical, e.g. medium composition (Main et al., 1967) and physical ones, e.g. temperature of cell culturing and time of blood collection (Ashman and Nalamas, 1977; Bijnen et al., 1979; Schultz and Adams, 1978; Pakin, 1977; Shifrine et al., 1978).

In this paper the influence of culture conditions on the reactivity of dog and fox lymphocytes is reported.

Materials and methods

Animals. Silver foxes used as blood donors were housed on a breeding farm and were maintained on a standard diet according to Scandinavian rules.

Bloodhound dogs were obtained from a private dogbreeder. Before beginning the experiment, faecal examination for parasites and haematological and physical examinations were performed on 10 breeding foxes (2–3 years old) and 10 Bloodhound dogs (3–5 years old). The animals used for this study appeared clinically normal.

Lymphocyte separation. Approximately 10 ml of blood was collected in a syringe containing 250 units of free heparin as preservative. Heparinized blood was diluted 1 : 1 with Hanks' solution and centrifuged for 30 min at 400 *g* and 22 °C in Ficoll/Uropolinum (Polfa) gradient (d = 1.075 g/cm^3) according to Boyum (1976). Mononuclear cells from the interphase were collected, washed twice with Hanks' solution and resuspended in Eagle's medium. This population contained more than 90% lymphocytes, as checked on the basis of May-Grünwald–Giemsa staining. The calculated recovery of lymphocytes was about 80%. Cell viability was 95% as assessed by the trypan blue exclusion test.

Lymphocyte stimulation test (LST). 2×10^5 cells per well were cultured on 96-well round-bottom tissue culture microplates (Sterilin, Great Britain) in a total volume of 0.2 ml of Eagle's medium, supplemented with gentamycin (20 μg/ml), 2.5–20% heat-inactivated fetal calf serum (FCS) and dog homologous serum (DHS) or fox homologous serum (FHS). Homologous serum was pooled serum obtained from 10 clinically healthy dogs and 10 healthy foxes. It was inactivated at 56 °C for 30 min and kept in 1 ml volumes at 20 °C until used. Leucoagglutinin (LA, Pharmacia), pokeweed mitogen (PWM, Gibco) and concanavalin A (Con A, Pharmacia) were used as mitogen. The extreme values of mitogens in foxes and dogs were as follow: Con A: 80,650–238,456 cpm; LA: 20,385–41,680 cpm; PWM: 21,456–39,860 cpm (foxes); Con A: 85,467–186,759 cpm; LA: 74,486–156,641 cpm; PWM: 23,450–49,860 cpm (dogs). All microcultures were performed in triplicate. Cells were cultured either at 37 °C or at 39 °C in humidified atmosphere containing 5% CO_2. Six h before harvest 1 μCi (37 kBq) of ^{3}H-Tdr (UVVR, Czechoslovakia) per well was added. Cells were collected on glass fibre filters and washed 3 times with water. Radioactivity was quantitated in a liquid scintillation counter. The results were expressed as count per minute (CPM) and the stimulation index (SI) was calculated by dividing the mean CPM of triplicate cells by the mean CPM of triplicate controls. CPM was calculated according to Barta (1984).

Results

The effect of varying concentrations of Con A, LA and PWM on the blastogenesis of dog lymphocytes was tested using a 3-day incubation period (Fig. 1a) and a 2-day culture of fox lymphocytes (Fig. 1b). In dogs, the optimal doses of mitogens were 10 μg/ml for Con A, 5 μg/ml for LA and 1:25 dilution

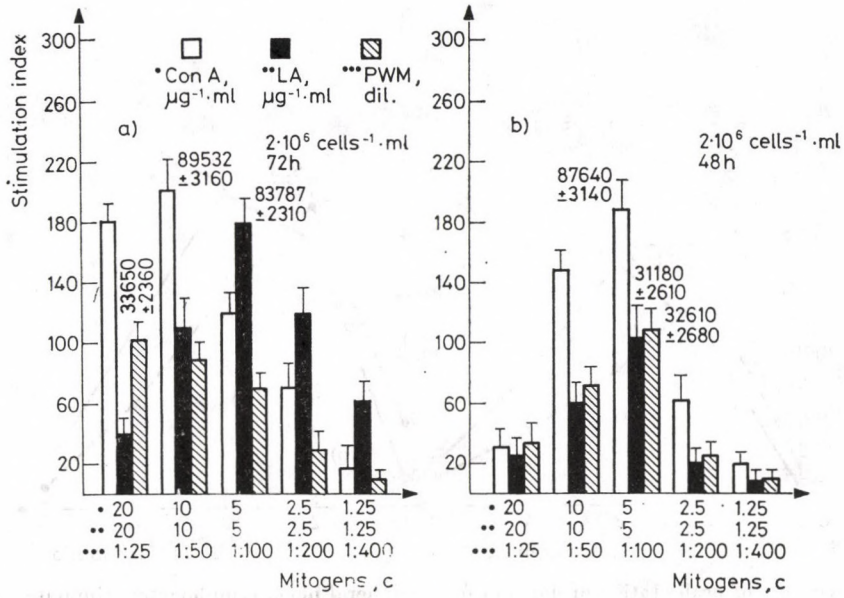

Fig. 1. Stimulation indices (SI) in dog peripheral blood lymphocytes cultured for 3 days (a) and in fox peripheral blood lymphocytes cultured for 2 days (b) with various doses of concanavalin A (Con A), leucoagglutinin (LA) or pokeweed mitogen (PWM). Each bar represents mean values ± S.D. from 10 experiments. Numbers adjacent to the bars are the mean values of CPM + S.D.

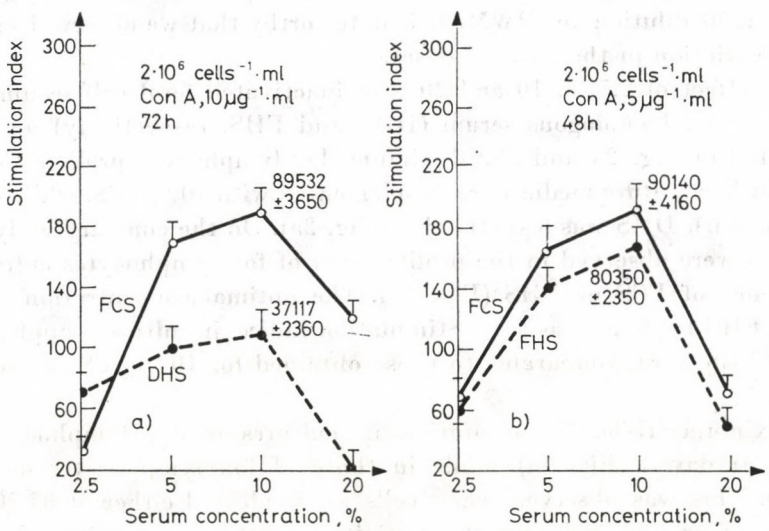

Fig. 2. Stimulation indices (SI) in dog peripheral blood lymphocytes cultured for 3 days (a) and in fox peripheral blood lymphocytes cultured for 2 days (b) with optimal doses of concanavalin A (Con A) in media supplemented with various concentrations of fetal calf serum (FCS) and dog homologous serum (DHS) or fox homologous serum (FHS). Both lymphocyte cultures were incubated at 39 °C. Each point represents the mean values of stimulation indices (SI) ± S.D. from 10 experiments

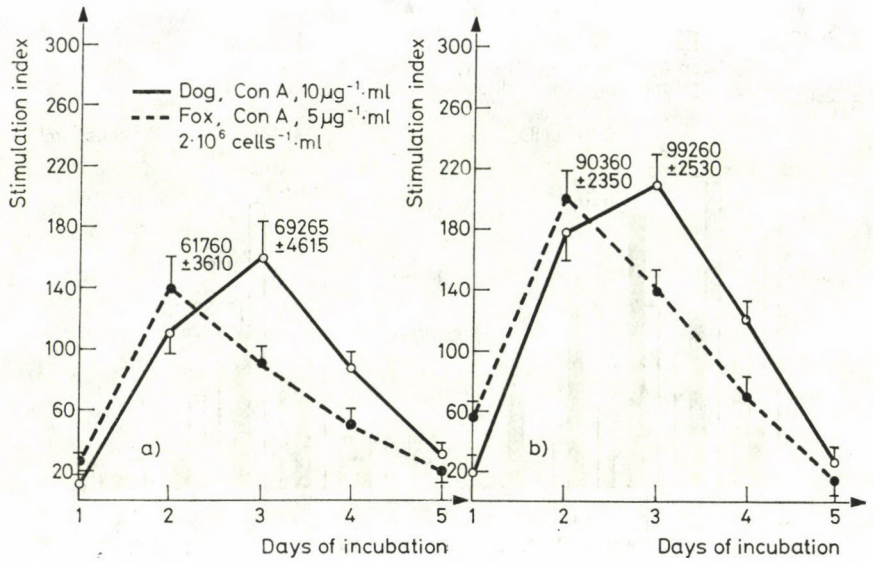

Fig. 3. Kinetics of proliferation of dog and fox peripheral blood lymphocytes stimulated with optimal doses of concanavalin A (Con A). Lymphocyte cultures were incubated at 37 °C (a) and at 39 °C (b). Each point represents mean values of stimulation indices (SI) ± S.D. from 10 experiments

for PWM, while in foxes the optimal doses were 5 μg/ml for Con A, 5 μg/ml for LA and 1:100 dilution for PWM. It is noteworthy that we observed some individual variation in the optimal doses.

The effect of 2.5, 5, 10 and 20% of inactivated fetal calf serum (FCS) and dog or fox homologous serum (DHS and FHS, respectively) on the SI is presented in Fig. 2a and 2b. Maximum dog lymphocyte proliferation was observed when culture media were supplemented with 10% FCS, while supplementation with DHS was less effective (Fig. 2a). On the contrary, only slight differences were observed in the proliferation of fox lymphocytes cultured in the presence of FCS or FHS (Fig. 2b). The optimal concentration of both FCS and FHS for foxes was 10%. Stimulation indices in cultures supplemented with 5% FCS were comparable to those obtained for 10% FCS in both dog and fox.

Maximum ^{3}H-Tdr incorporation in cultures of dog lymphocytes was observed at day 3 (Fig. 3a), while in those of fox lymphocytes on day 2 (Fig. 3b). This was observed when cells were cultured either at 37 °C or at 39 °C. However, cells cultured at 39 °C incorporated more tritiated thymidine in the majority of animals tested than did cells cultured at 37 °C for the same time.

Discussion

The results show that dog and fox lymphocytes have dissimilar requirements for culture conditions, although both species belong to the same family of Carnivora. In dogs, the highest lymphocyte proliferation was observed in most cases at day 3 for all mitogens, but in some individuals high thymidine incorporation was observed also at day 2. Thus, the plateau of response could reach 24 h. This may explain some differences in the kinetics of lymphocyte proliferation estimated by other authors. Betton et al. (1980) observed maximum proliferation of culture on days 3–4 and days 4–5 for Con A and PHA, respectively. Barta et al. (1983) observed the highest ^{3}H-Tdr incorporation at day 3 for Con A, PHA and PWM as well. The kinetics of *in vitro* lymphocyte proliferation may be affected by numerous factors including the time of blood collection, circannual rhythm, the age of animals and the culture conditions (Shifrine et al., 1978; Gerber and Brown, 1974; Main et al., 1967). Direct comparison between results obtained in different laboratories is difficult. The genetic variation in lymphocyte response among different breeds should be also taken into consideration. In the present experiments, only Bloodhound dogs were used, so the results are partially comparable with those reported by others.

The highest ^{3}H-Tdr incorporation in cultures of fox lymphocytes was observed at day 2, and it usually came down to at least half by day 3. Our results differ from those of Janot et al. (1982) who reported very low SI for cultures of red fox lymphocytes. This discrepancy may be attributed to the fact that the latter authors measured ^{3}H-Tdr incorporation at day 4 or 5.

The SI of dog peripheral lymphocytes to the mitogens Con A and LA was in similar range and was usually higher for Con A than for LA. On the other hand, a slight response was observed to PWM, similarly to data reported by others (Barta et al., 1983; Shifrine et al., 1978).

The blastogenesis of fox lymphocytes was greater in Con A stimulated cultures. LA and PWM showed weaker blastogenesis than Con A, but still had a significant stimulatory action with no essential difference between them. It is worth noting that only one paper discussing this problem has been found (Janot et al., 1982). Time differences in quantity measurements of incorporated thymidine do not allow a comparison of our results with those obtained by others.

The importance of serum in *in vitro* cultures of various species has been reported by many authors (Barta et al., 1982; Barta, 1984; Kristensen et al., 1982; Kok-Mun Than et al., 1982). Bloodhound dogs showed a markedly better proliferative response in the presence of fetal calf serum (FCS), while the proliferation of fox lymphocytes in media supplemented with FCS was comparable to that seen in media supplemented with homologous serum (FHS). This

supports the conclusion drawn by Barta (1984) on the necessity of testing supplementing sera of various origin in each experimental model to obtain a maximum lymphocyte response. FCS is most commonly employed for the supplementation of lymphocyte cultures (Barta, 1984; Kok-Mun Than et al., 1982; Kristensen et al., 1982). Holt et al. (1966), Hayry and Defendi (1970) and Barta et al. (1982) reported that lymphocyte responsiveness varies by species from which the serum supplement is derived.

Some authors reported better responsiveness to mitogens of whole blood than of purified lymphocyte cultures (Kok-Mun Than et al., 1982; Kristensen et al., 1982; Shifrine et al., 1978). In our experiments, the stimulation indices obtained for cultures of purified lymphocytes were fully comparable to indices reported by other authors for whole blood cultures.

The results presented in this paper indicate that estimation of lymphocyte proliferation in animals of various, even closely related, species always requires a careful checking of culture conditions which strongly influence the range of response. Thus, the optimization of culture conditions should be the first step of any investigation.

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EFFECT OF T-2 TOXIN ON EGG PRODUCTION AND HATCHABILITY IN LAYING HENS

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The effect of diets containing different levels of T-2 toxin on egg production and hatchability was studied in a four-week experiment using 100 laying hens of the SSL hybrid line and 10 cocks divided into 10 groups. Another aim of the experiment was to investigate how effectively the increased dietary vitamin E content neutralized the adverse effects of T-2 toxin.

The diet of the control group (C) contained no mycotoxin, while those of the experimental groups included the following levels of T-2 toxin: groups 1, 2 and 3: 1 mg/kg, groups 4, 5 and 6: 5 mg/kg; groups 7, 8 and 9: 10 mg/kg. Vitamin E was added to the diet of groups C, 1, 4 and 7 at a rate of 50 mg/kg while to that of groups 2, 5 and 8 at a rate of 100 mg/kg. To the diet of groups 3, 6 and 9 no vitamin E was added.

Contamination of the diet with T-2 toxin markedly decreased egg production and impaired hatchability. The production decrease was proportional to the T-2 toxin concentration of the diet.

Increased dietary vitamin E concentration exerted no influence on egg production. However, during the first week of the experiment it significantly ($P < 0.01$) decreased the number of infertile eggs and significantly ($P < 0.01$) improved the hatching percentage. Dietary vitamin E concentration was in positive correlation with the hatching percentage; this correlation was rather close ($r = 0.74$) in the first week of the experiment.

Key words: T-2 toxin, diet, chicken, egg production, hatchability, vitamin E

The contamination of animal feeds by mycotoxins has enormous economic, scientific and public health significance. Mycotoxins increase the mortality rate, reduce the body mass gain and the egg yield, and substantially impair the feed conversion rate, too. From the public health point of view, it is the cumulative character of mycotoxins which is of great concern, i.e. that they accumulate in the inner organs of animals, become incorporated in eggs or excreted with the milk. If we add moreover that some mycotoxins (e.g. aflatoxins) have a carcinogenic effect, we can see how big human health risks their presence in animal feeds involves.

Data available in the special literature suggest that the number of existing fungus species is around 100,000. Of them, 200–300 are known to produce mycotoxins. Up to this day, more than 300 mycotoxins have been isolated and their chemical composition has been determined.

As a result of the geographical situation and climatic conditions characteristic of our region, the toxins produced by *Fusarium* species have great

sanitary and economic importance. Maize often becomes contaminated by fusaria before it is gathered in. If the essential conditions (humidity, suitable temperature, oxygen) are present, these fusaria will continue to grow, multiply and produce mycotoxins in the barns, silos and storehouses.

It is generally accepted that fusariotoxins can be divided into two groups. The first group comprises zearalenone, a toxin of oestrogenic effect, and its metabolites like alpha- and beta-zearalenol, while the second group includes the trichothecene mycotoxins. About 80% of the fusariotoxins known at present belong to the second group. Due to the great health hazards associated with them, the most important members of the group are T-2 toxin and its metabolites, DAS, DON, nivalenol and fusarenon-X.

As there is a scarcity of data on the effects of T-2 toxin in the Yugoslav literature, this experiment was designed to reveal the negative effects, if any, exerted by T-2 toxin on egg production and hatchability in laying hens, and to determine how effectively vitamin E supplementation of the diet neutralizes the adverse effects of T-2 toxin.

Materials and methods

One hundred laying hens of the hybrid line SSL (age: 28 weeks) and 10 cocks were divided into 10 groups. The hens of all groups were given the same layer's diet throughout the 4-week experimental period, with the difference that the diet of the control group (C) was free from mycotoxin while to that of the experimental groups T-2 toxin was added at the following dose rates: 1 mg/kg (groups 1, 2 and 3), 5 mg/kg (groups 4, 5 and 6), and 10 mg/kg (groups 7, 8 and 9).

T-2 toxin was produced in our laboratory, using *Fusarium tricinctum* syn. *sporotrichioides* (Bata et al., 1983) and was identified and quantitated by the capillary chromatographic method described earlier (Ványi et al., 1982; Bata et al., 1983).

As the mineral and vitamin supplement did not contain vitamin E, the vitamin E content of the diet equalled that of the basic materials (11 mg/kg). The diet of the control group (C) and of groups 1, 4 and 7 was supplemented with 50 mg/kg while that of groups 2, 5 and 8 with 100 mg/kg vitamin E. No vitamin E was added to the diet of groups 3, 6 and 9.

The egg production of the different groups was monitored daily throughout the experiment. Eggs suitable for hatching were placed in a "Pas Reform" type incubator (eggs produced by the different groups were incubated separately). Infertile eggs were sorted out by candling on day 8 of incubation. The number of viable chicks that had hatched out was recorded at the end of the incubation period.

Results

The weekly egg production of the different groups of laying hens is presented in Table 1. For the entire period of experiment, average egg yield of the control group was 91.43%. Contamination of the experimental groups' diet by T-2 toxin markedly decreased the egg yield: this drop in egg production was proportional to the dietary concentration of T-2 toxin. While in groups 1, 2 and 3 fed 1 mg/kg T-2 toxin egg production dropped to 74.29, 79.64 and 80.00%, respectively, in groups 4, 5 and 6, in which the dietary concentration of the toxin was 5 mg/kg, egg production decreased to 27.87, 36.43 and 29.29%, respectively. Addition of T-2 toxin to the diet at a dose rate of 10 mg/kg (groups 7, 8 and 9) resulted in egg production drops to levels as low as 18.57, 21.79 and 19.29%, respectively.

The weekly egg yield showed the same tendency as did the average egg yield calculated for the entire period of experiment, i.e. that the egg production drop was proportional to the T-2 concentration of the layer's diet. The decrease in egg production was the most expressed in the third and fourth week and the least expressed in the first week.

According to the statistical calculations (Table 2), addition of T-2 toxin to the diet resulted in a significant ($P < 0.05$; groups 4 and 6) and highly significant ($P < 0.01$; groups 5, 7, 8 and 9) egg production drop already in the first week of the experiment. In the fourth week of the experiment, the production drop was highly significant ($P < 0.01$) in all experimental groups with respect to the control group.

A close negative correlation ($r = -0.93$) was found to exist between egg production and dietary T-2 toxin concentration. In contrast to this, a very low positive correlation ($r = 0.04$) was established between dietary vitamin E concentration and egg production.

The deleterious effect of T-2 toxin is clearly reflected by the hatching results as well (Tables 3 and 4). The most pronounced adverse effect was demonstrable in the period between day 15 and 28, when a significant ($P < 0.01$) negative correlation ($r = -0.86$) existed between dietary T-2 concentration and the hatching percentage. In all other periods studied as well as for the entire experiment taken as a whole, this negative correlation was low and nonsignificant ($P > 0.05$).

From Table 3 it appears that vitamin E supplementation of the diet of groups 1, 4 and 7 (50 mg/kg) and groups 2, 5 and 8 (100 mg/kg) considerably increased the hatching rate as compared to groups 3, 6 and 9 which received a diet not supplemented with vitamin E. This increase was the biggest in the period between days 1 and 9 and the smallest in that between day 15 and 28.

Both the T-2 toxin and the vitamin E concentration of the diet markedly influenced the percentage of infertile eggs (Table 4). Parallel with increasing

Table 1

Egg production of the different groups of laying hens during the experiment

Groups	C	I	II	III	IV	V	VI	VII	VIII	IX
T-2 toxin, mg/kg	—	1	1	1	5	5	5	10	10	10
Vitamin E, mg/kg	50	50	100	–	50	100	–	50	100	–
					Days 1–7					
Eggs, pcs	65	62	65	58	51	50	51	40	47	50
Egg yield, %	92.86	88.57	92.86	82.86	72.86	71.43	72.86	57.14	67.14	71.43
Index	100	95.38	100	89.23	78.46	76.92	78.46	61.54	72.30	76.92
					Days 8–14					
Eggs, pcs	64	54	55	59	22	29	21	10	10	3
Egg yield, %	91.43	77.14	78.57	84.29	31.43	41.43	30.00	14.29	14.29	4.29
Index	100	84.38	85.94	92.19	34.37	45.31	32.81	15.63	15.63	4.69
					Days 15–21					
Eggs, pcs	59	42	51	51	0	13	6	2	2	2
Egg yield, %	84.29	60.00	72.87	72.87	0	18.57	8.57	2.86	2.86	0
Index	100	71.86	86.44	86.44	0	22.03	10.17	3.39	3.39	0
					Days 22–28					
Eggs, pcs	68	50	52	56	5	10	4	0	2	1
Egg yield, %	97.14	71.43	74.29	80.00	7.14	14.29	5.71	0	2.86	1.43
Index	100	73.53	76.48	82.35	7.35	14.71	5.88	0	2.94	1.47
					Days 1–28					
Eggs, pcs	256	208	223	224	78	102	82	52	61	54
Egg yield, %	91.43	74.29	79.64	80.00	27.87	36.43	29.29	18.57	21.79	19.29
Index	100	81.25	87.10	87.50	30.48	39.84	32.04	20.32	23.83	21.10

Table 2

Statistical evaluation of the daily egg yield

Group	2	X	±SX	SD	CV	IV
1	2	3	4	5	6	7
			Days 1–7			
C	10	9.28	0.221	0.70	7.53	8–10
I	10	8.85	0.313	0.99	11.17	8–10
II	10	9.28	0.221	0.70	7.53	8–10
III	10	8.28	0.405	1.28	15.42	6–10
IV	10	7.28*	0.525	1.66	22.87	4–9
V	10	7.14**	0.573	1.81	25.29	4–9
VI	10	7.28*	0.468	1.48	20.38	5–10
VII	10	5.71**	0.788	2.49	43.61	2–9
VIII	10	6.71**	0.579	1.83	27.26	4–9
IX	10	7.14**	0.687	2.17	30.34	4–10
			Days 8–14			
C	10	9.14	0.313	0.99	10.83	7–10
I	10	7.71	0.424	1.34	17.38	5–10
II	10	7.85	0.490	1.55	19.74	6–9
III	10	8.42	0.373	1.18	14.01	6–10
IV	10	3.14**	0.573	1.81	57.64	1–6
V	10	4.14**	0.427	1.35	32.61	3–7
VI	10	3.00**	0.446	1.41	47.00	1–5
VII	10	1.42**	0.285	0.90	63.38	0–3
VIII	10	1.42**	0.474	1.50	105.63	0–4
IX	10	0.42**	0.155	0.49	116.67	0–1
			Days 15–21			
C	10	8.42	0.598	1.89	22.45	4–10
I	10	6.00**	0.237	0.75	12.50	5–7
II	10	7.28*	0.405	1.28	17.58	6–9
III	10	7.28*	0.278	0.88	12.09	6–8
IV	10	0.00**	0.000	0.00	0.00	0–0
V	10	1.85**	0.392	1.24	67.65	0–2
VI	10	0.85**	0.263	0.83	97.65	0–2
VII	10	0.28**	0.142	0.45	160.71	0–1
VIII	10	0.28**	0.142	0.45	160.71	0–1
IX	10	0.00**	0.000	0.00	0.00	0–0
			Days 22–28			
C	10	9.71	0.142	0.45	4.63	9–10
I	10	7.14**	0.313	0.99	13.86	6–9
II	10	7.42**	0.332	1.05	14.15	6–9
III	10	8.00**	0.237	0.75	9.37	7–9
IV	10	0.71**	0.221	0.70	98.59	0–2
V	10	1.42**	0.231	0.73	51.41	0–2
VI	10	0.57**	0.231	0.73	128.07	0–2
VII	10	0.00*	0.000	0.00	0.00	0–0
VIII	10	0.28**	0.452	1.43	510.71	0–2
IX	10	0.14**	0.111	0.35	250.00	0–1

Table 2 continued

Group	n	X	±SX	SD	CV	IV
1	2	3	4	5	6	7
			Days 1–28			
C	10	9.14	0.392	1.24	13.57	4–10
I	10	7.43**	0.465	1.47	19.07	5–10
II	10	7.96	0.427	1.35	1.69	5–10
III	10	8.00	0.358	1.13	14.12	6–10
IV	10	2.79**	0.987	3.12	111.83	0–9
V	10	3.64**	0.832	2.63	72.75	0–9
VI	10	2.93**	0.927	2.93	100.00	0–10
VII	10	1.86**	0.839	2.65	142.47	0–9
VIII	10	2.18**	0.930	2.94	134.86	0–9
IX	10	1.93**	1.019	3.22	166.84	0–10

* significant difference ($p < 0.05$); ** highly significant difference ($p < 0.01$)

Table 3

Hatching percentages

Group	Periods				
	Days 1–7	Days 8–14	Days 15–28	Days 1–28	Index
C	87.5	92.5	80.0	86.6	100
I	87.5	86.6	40.0	70.0	80.83
II	87.5	80.0	52.0	66.4	76.67
III	77.5	53.3	35.0	55.5	64.09
IV	90.0	52.4	0	71.2	82.22
V	92.5	82.8	26.0	72.8	84.06
VI	77.5	66.7	10.0	64.8	74.83
VII	80.0	80.0	0	76.9	88.80
VIII	82.5	55.5	0	71.7	82.80
IX	72.5	33.3	0	45.5	52.54

Table 4

Infertile eggs, %

Group	Period				
	Days 1–7	Days 8–14	Days 15–28	Days 1–28	Index
C	5.0	2.5	7.5	5.00	100
I	7.5	6.7	50.0	22.73	454.6
II	5.0	13.3	45.0	21.82	436.4
III	17.5	23.3	55.0	32.73	654.6
IV	2.5	33.3	80.0	18.18	363.6
V	2.5	13.8	60.9	20.65	413.0
VI	15.0	23.8	80.0	26.76	535.2
VII	15.0	10.0	100.0	17.31	346.2
VIII	10.0	22.2	100.0	18.87	377.4
IX	17.5	33.3	100.0	20.45	409.0

dietary T-2 toxin concentration, the number of infertile eggs substantially increased. This tendency was most expressed in the period between days 15 and 28 and least expressed in that between days 1 and 7.

The omission of vitamin E supplementation of the diet (groups 3, 6 and 9) resulted in increased percentages of infertile eggs. This increase was highly significant ($P < 0.01$) in the period between days 1 and 7.

Discussion

The results of the 4-week experiment clearly indicate that the addition of T-2 toxin to the diet of laying hens substantially decreased the egg yield and impaired the hatching results.

Contamination of the diet with T-2 toxin at a dose level as low as 1 mg/kg decreased the average egg yield in groups 1, 2 and 3 by 18.75, 12.90 and 12.50%, respectively, with respect to the control group. The egg yield of groups 4, 5 and 6, fed a diet containing 5 mg/kg T-2 toxin, decreased by 69.52, 60.16 and 67.96%, respectively. The lowest egg yield was recorded in groups 7, 8 and 9 fed 10 mg/kg T-2 toxin in the diet: the decrease was 79.69, 76.17 and 78.90%, respectively, as compared to the egg production of group C.

The results obtained in this study are consistent with those published in the literature. Several authors reported that T-2 toxin causes an egg production drop in laying hens (Speers et al., 1971; Wyatt et al., 1975; Hamilton, 1984; Hofacre et al., 1985; Ványi, 1986; Kralj et al., 1987).

The egg production drop occurring in T-2 toxicosis can probably be accounted for by feed refusal and low daily feed intake. Namely, lowered feed intake results in the ingestion of less protein and other nutrients essential for egg production. T-2 toxin is known to inhibit DNA and protein synthesis in the cells. If we add moreover that the toxin can induce severe atrophy of the liver and the genital organs in a short time (Palyusik et al., 1981; Tobiaš, 1990), its role in the aetiology of dropped egg production can easily be understood.

The poor hatchability caused by T-2 toxin can primarily be attributed to the increased number of infertile eggs. Several authors (Palyusik et al., 1971; Wolf, 1987; Ványi, 1989) reported the adverse effect exerted by T-2 toxin on the fertilization of hatching eggs.

The proportion of infertile eggs was the highest and the hatching percentage was the lowest between days 15 and 28 of the experiment, a finding which indicates the cumulative property of T-2 toxin.

Elevated vitamin E concentrations of the diet did not affect egg production but markedly decreased the number of infertile eggs and, consequently, caused a pronounced improvement in the hatching percentage. This effect

was the most pronounced in the first week of the experiment when vitamin E significantly ($P < 0.01$) improved the hatching results. Later on, after T-2 toxin had cumulated, it largely neutralized the growth-promoting effect of vitamin E. Thus, in these periods the reduction in the number of infertile eggs and the increase in the hatching percentage caused by vitamin E was no longer significant ($P > 0.05$).

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FURTHER MONOGENEANS FROM IRANIAN FRESHWATER FISHES

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Thirteen *Dactylogyrus*, 1 *Dogelius* and 1 *Tetraonchus* species are first recorded on Iranian fishes. Of them, twelve *Dactylogyrus* species and *Tetraonchus monenteron* are identified with known species. *Dogelius persicus* sp. n. from *Barbus sharpeyi*, *Barbus grypus* and *Carassobarbus luteus*, as well as *Dactylogyrus holciki* from *Chalcalburnus mossulensis* and *C. chalcoides* are described as new species. Besides the first record of the above monogeneans, new host records are given to some known species.

Key words: Monogenea, freshwater fishes, *Dactylogyrus*, *Dogelius*, *Tetraonchus*, host, Iran

Monogeneans from Iranian fishes were first described by Bychowsky (1949) who reported on the occurrence of three *Dactylogyrus* and an *Ancyrocephalus* species on the gills of fishes in River Karkheh. Further monogeneans were recorded by Jalali and Molnár (1990*a*, *b*) who found 24 species on fishes from natural waters and 14 species on fishes cultured in pond farms. Among the species found they identified 22 known *Dactylogyrus* spp. and described a new *Dogelius* sp.

The detection of further *Dactylogyrus* spp. new for Iran is reported and a new *Dactylogyrus* sp. and *Dogelius* sp. are described in this paper.

Materials and methods

Fishes were collected in three different geographical regions of Iran, namely from the rivers Sefid, Tonekabon and Tajan running to the Caspian Sea, the rivers Dez, Karun and Beshar belonging to the Persian Gulf system, and from the rivers Zayandeh, Kor and Jaj flowing into sodium lakes in Central Iran. In addition, some fish were collected from Lake Sama in the Elburz Mountain (Table 1).

More than 600 fish specimens were examined. However, only those fish were recorded which on their gills and fins harboured monogeneans which were identified to species (Tables 1 and 2).

Table 1

First record of known monogenean parasites on Iranian fishes

No.	Name of parasite	Name of host	Locality		
			Ponto-Caspian region	Persian Gulf region	Inland watersheds
1.	*Dactylogyrus affinis* Bychowsky, 1933	*Barbus brachycephalus*	River Sefid		
2.	*D. chramulii* Kojava, 1966	*Capoeta capoeta*	River Sefid	River Beshar	
3.	*D. carpathicus* Zachvatkin, 1951	*Barbus plebeius*	River Sefid		
4.	*D. gracilis* Mikailov, 1974	*Capoeta capoeta*	River Sefid		River Zayandeh
5.	*D. intermedius* Wegener, 1910	*Carassius auratus*			Petfish farm close to Tehran
6.	*D. lenkorani* Mikailov, 1967	*Capoeta capoeta*	River Sefid Tajan Tonekabon	River Beshar	River Zayandeh, Kor
7.	*D. linstowi* Bychowsky, 1936	*Barbus plebeius* *Barbus capito*	River Sefid River Sefid		
8.	*D. minor* Wagener, 1857	*Chalcalburnus chalcoides*			River Ghasemlu
9.	*D. parvus* Wegener, 1910	*Alburnus charusini*	River Sefid		
10.	*D. suecicus* Nybelin, 1937	*Rutilus frisii kutum*	River Sefid		
11.	*D. tuba* Linstow, 1878	*Aspius aspius*	River Sefid		
12.	*D. vistulae* Prost, 1957	*Ch. chalcoides*	River Sefid		River Ghasemlu
13.	*Tetraonchus monenteron* Wagener, 1857	*Esox lucius*			Lake Sama

Results

The majority of fish examined in this study had monogenean infestation of the gills. Of the monogeneans, twelve *Dactylogyrus* spp. and a *Tetraonchus* sp. proved to be known species and were identified with monogeneans of the Ponto-Caspian fauna region (Table 1). These parasites had been collected from 19 host species. Besides monogeneans detected for the first time during this survey, *Dactylogyrus* spp. recorded earlier (Bychowsky, 1949; Jalali and Molnár, 1990*a*) were frequently encountered. Of them, those which were found in new hosts are presented in Table 2.

Table 2

Monogenean species known from Iran but found in hosts other than previously recorded

No.	Name of parasite	New hosts	Locality	Hosts by Jalali and Molnár (1990*a*)
1.	*Dactylogyrus alatus* Linstow, 1878	*Chalcalburnus chalcoides*	River Ghasemlu	*Alburnoides bipunctatus*
		Alburnus charusini	River Sefid	
2.	*D. chalcalburni* Dogiel and Bychowsky, 1934	*Chalcalburnus chalcoides*	River Sefid River Zayandeh	*Alburnoides bipunctatus* *Alburnus alburnus*
		Alburnus charusini	River Sefid	
3.	*D. haplogonus* Bychowsky, 1933	*Rutilus frisii kutum*	River Sefid	*Vimba vimba persa*
4.	*D. pulcher* Bychowsky, 1957	*Capoeta capoeta*	River Ghasemlu River Jaj Tonekabon Sefid Tajan	*Aspius vorax*
		Capoeta trutta	River Dez	
5.	*D. turaliensis* Aligadzhiev, Gussev, Kazieva, 1984	*Rutilus frisii kutum*	River Sefid	*Rutilus rutilus caspicus*

In addition to the known species, several undescribed new monogeneans were found on fishes of the Persian Gulf basin and of rivers running into the sodium lakes of Central Iran. Most of the latter species require further studies for a detailed description. Therefore, only a *Dactylogyrus* sp. and a *Dogelius* sp. is described here. Both parasites occurred exclusively on the gills. All measurements are given in μm.

Dactylogyrus holciki n. sp. (Fig. 1)
Hosts: *Chalcalburnus mossulensis* (Heckel) and *C. chalcoides* Güldenstadt
Locality: River Beshar

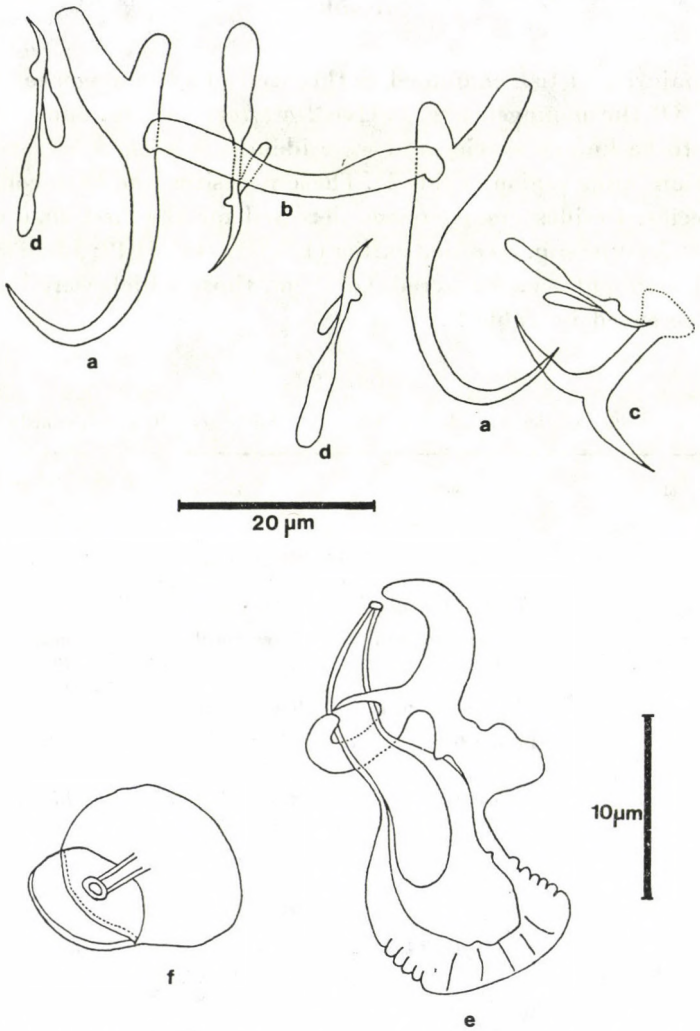

Fig. 1. Sclerotized organs of *Dactylogyrus holciki.* a) anchors; b) dorsal bar; c) ventral bar; d) hooks; e) copulatory organ; f) vagina

Specimens studied: 9. Type specimen from *C. mossulensis* has been deposited in the collection of the Zoological Department, Hungarian Natural History Museum, Budapest.

Medium-sized *Dactylogyrus.* Body 850 (720–950) long and 110 (90–126) wide. Hooks of different size with massive handle and with well-projected heel of blades. Their length 16.5–21.5 (the smallest), 23–28 (the biggest). Anchors slender. Ventroapical length of anchors 34 (28–36.5), dorsoapical length 35

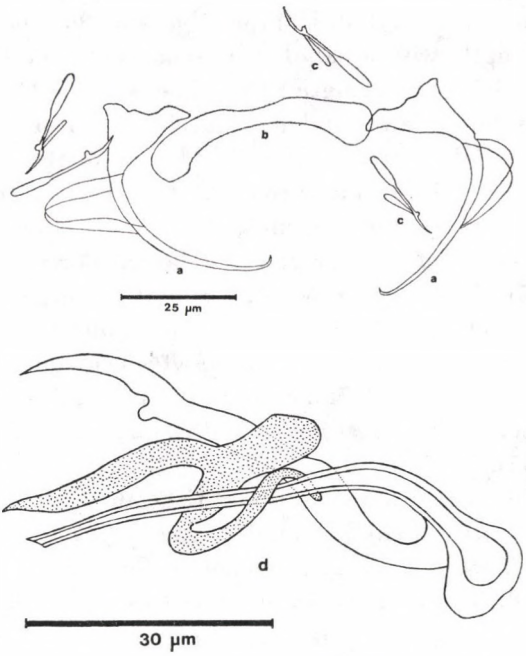

Fig. 2. Sclerotized organs of *Dogelius persicus.* a) anchors; b) bar; c)hooks; d) copulatory organ

(30–36.5), main part 29 (26.5–31.5), tip 9 (8.3–11). Inner root 10 (8.3–12), outer root 5.3 (4.4–5.8). Dorsal bar thin, elongated and slightly bent backwards, 3.4 (2.5–4.2) long and 26.5 (20–31.5) wide. Ventral bar triangular, with an anteriorly and two laterally directed processes. The latter enlarged at the end. Length 11.3 (10–13), width 21.5 (16.5–26.5). Copulatory organ composed of a short thick tube, a strong initial part and a triangular accessory piece. One of the processes of the accessory piece surrounds the tube as a pincers. Length 37.5 (33–40). Sclerotized vagina composed of two round discs with a short tube between them. It measures 19.5 (15–25).

This species differs from all known *Dactylogyrus* species by the shape of its copulatory organ. The species is named in the honour of Dr. Juraj Holcik, a well-known Slovak ichthyologist and ecozoologist.

Dogelius persicus n. sp. (Fig. 2)

Hosts: *Barbus grypus* Heckel, *B. sharpeyi* Günter and *Carassobarbus luteus* (Heckel)

Locality: Rivers Dez and Karun

Specimens studied: 6. Type specimen from *Barbus grypus* has been deposited in the collection of the Zoological Department, Hungarian Natural History Museum, Budapest.

Body moderately elongated. Haptor subquadrate. Four eyespots present. Hooks similar in length, with a handle well separated from the pivot and with well-projected heel of blade. Length of the hooks 23.5 (23–25). Anchors with relatively well-developed inner and less developed outer roots. Point of the anchors without special blades. Ventroapical length of the anchors 52 (50–60), dorsoapical length 40 (35–51), inner root 12 (11.5–13), outer root 5. Only one bar is present. It is enlarged at the ends, thickened in the centre and slightly bent anteriorly. Length of the bar at the enlarged portion 9.5 (8–11), at the middle 5.5 (5–6.5). Width 49 (46–53). Copulatory organ composed of an elongated tube, an elongated basal part which ends in a blade and of an accessory piece having three processes, one of which surrounds the tube. Length of the copulatory organ 60 (43–72). Sclerotized vagina is built of a sphaerical disc and a short bent tube. The diameter of the disc 21 (17–26), the length of the tube 23 (17–35).

Specimens found in different barboid fishes had identical morphological characteristics but differed slightly in size. The biggest specimens were found in *Barbus sharpeyi*, while the smallest ones in *Carassobarbus luteus*.

This species differs from the known *Dogelius* species by the construction of the copulatory organ and by lacking the special blade on the tip of the anchors.

Discussion

Data obtained in this survey and those obtained in earlier studies (Bychowsky, 1949; Jalali and Molnár, 1990*a*, *b*) indicate that, similarly to the composition of fish species (Berg, 1940; Coad, 1979), the monogenean fauna of Iranian fishes varies by habitat. *Dactylogyrus* spp. infecting fishes of the Ponto-Caspian region correspond to species known from the Palaearctics; however, those infecting fishes endemic in the Mesopotamian region represent mostly unknown species. In the latter habitat known *Dactylogyrus* spp. were found only on introduced fishes and on some very common fishes like *Capoeta capoeta*, which are widespread in this region but cannot be regarded as original inhabitants of these waters.

The species composition of both fishes and monogeneans suggests that the fauna of rivers running into sodium lakes in Central Iran belongs to, or is very close to, the Ponto-Caspian region.

Dactylogyrus spp. of European and East-African fishes show very strict host specificity and mostly infect only one fish species (Gussev, 1985; Molnár et al., 1984). *Dactylogyrus* species found on Iranian cyprinids exhibit less pronounced host specificity and infect some closely related fish species. In this way, *D. alatus* was detected on *Chalcalburnus*, *Alburnus* and *Alburnoides* spp., and *D. haplogonus* on *Vimba* and *Rutilus* spp.

Because of the relatively loose host specificity and the active or passive introduction of fishes to other fauna regions, the original monogenean fauna of different habitats cannot be monitored precisely. Still, it is suggested that *D. pulcher*, *D. chramuli* and *D. lenkorani*, which are typical Ponto-Caspian species, have only recently been introduced to the Mesopotamian region, while *D. holciki* n. sp. must have first been a parasite of *Chalcalburnus mossulensis* and only later on did it become an inhabitant of the gills of *C. chalcoides*.

Among species infecting goldfish, Jalali and Molnár (1990*b*) recorded 4 *Dactylogyrus* spp., among them *D. vastator forma minor*. This latter species was misidentified and its correct determination is given in this work as *D. intermedius* Wegener, 1910.

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HISTOENZYMIC EFFECTS OF THIOPHENATE AND FENBENDAZOLE ON THE ABSORPTIVE SURFACES OF *HAEMONCHUS CONTORTUS*

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In vitro alterations induced by a 10 μg/ml and 50 μg/ml dose each of thiophenate and fenbendazole on the absorptive surfaces of *Haemonchus contortus* (Nematoda: Trichostrongylidae) were studied. The most significant changes were induced in the gut epithelium. Alkaline phosphatase and adenosine triphosphatase activities were decreased, succinic dehydrogenase activity was increased, while acid phosphatase and glucose-6-phosphatase were completely lost from the intestinal epithelium after treatment with either of the drugs. A stimulatory effect of these two anthelmintics was observe on lactic dehydrogenase and reduced nicotinamide adenine dinucleotide diaphorase distribution. Thiophenate caused an increase in the activities of glutamate dehydrogenase (GDH), glucose-6-phosphate dehydrogenase (G-6-PD) and nonspecific esterases and a decrease in reduced nicotinamide adenine dinucleotide phosphate diaphorase (NADPH-D) activity. Fenbendazole treatment led to the inhibition of GDH, while G-6-PD, NADPH-D, cytochrome oxidase, monoamine oxidase and nonspecific esterase activity remained unaltered in the epithelium.

Key words: *Haemonchus contortus*, thiophenate, fenbendazole, absorptive surfaces

Haemonchus contortus (Rud., 1803) is one of the most pathogenic species parasitizing the abomasum of ruminants. It has attracted considerable attention by investigators in different parts of the world (see Sood and Kapur, 1982). During their absorption, anthelmintics may affect and/or modify the enzyme activity and may also alter the normal metabolism of the parasite's absorptive surfaces. The external surface and the gut are the two major areas through which absorption of the drugs can occur. The *in vivo* actions of anthelmintics regarding their efficacy have attracted considerable attention, but the mechanisms by which the drugs act on these two absorptive surfaces of the parasites are quite obscure. The biochemical differences between the host and the parasite form a basis for the selective toxicity of anthelmintics (Saz and Bueding, 1966). The present study is an attempt to elucidate the distribution of phosphatases, oxido-reductases and esterases and also to investigate the histoenzymic changes induced in the body wall and intestine by the *in vitro* application of two commonly used anthelmintics, thiophenate and fen-

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bendazole. Previously, similar studies using nilzan and albendazole (Kaur and Sood, 1990*a*) as well as nilverm and nilverm forte (Kaur and Sood, 1990*b*) have been done.

Materials and methods

Worms and drugs. Mature, motile worms of either sex were collected from the abomasa of goats *(Capra hircus)* slaughtered at local abattoirs. The worms were washed thoroughly with saline and freed from adhering host material. The collected worms were maintained in Tyrode's solution (Ward, 1974) at 37 °C. Two different concentrations (10 μg/ml and 50 μg/ml) each of thiophenate ethyl (thiophenate, wettable powder 70% w/w, May and Baker, India Ltd.) and fenbendazole (Panacur 25% powder, Pfizer, India) were prepared in Tyrode's solution.

Incubations. About 10 motile worms were incubated at 37 °C in 25 ml of Tyrode's solution (control) and each of the above-mentioned drug dilutions. About 50% mortality was achieved after 10 h of incubation in 10 μg/ml concentrations of either of the drugs, while all the parasites died within 6–8 h in 50 μg/ml of thiophenate as well as fenbendazole.

Histoenzymology. For histochemical localization of phosphatases, dehydrogenases, oxidases and esterases, cryostat sections of 10 μ thickness of nonincubated (normal, Tyrode-incubated) and drug-treated parasites were cut and stored at −20 °C. These sections were incubated for alkaline (AkPase), acid (AcPase), glucose-6 (G-6-Pase) phosphatases (Barka and Anderson, 1963), adenosine triphosphatase (ATPase), cytochrome (CYO) and monoamine (MAO) oxidases and nonspecific esterases (NSE, Chayen et al., 1969), acetylcholinesterase (AChEase; El-Badawi and Schank, 1967), succinic (SDH), lactic (LDH), glutamate (GDH) and glucose-6-phosphate (G-6-PD) dehydrogenases, and reduced nicotinamide adenine dinucleotide phosphate (NADPH-D) diaphorases (Pearse, 1972). The observations were recorded in different layers of the parasites and scored from negative (−) to very strong (++++).

Results

Phosphatases (Plate I). The complete loss of activity was detected in the contractile part of muscles after treatment with 10 μg/ml each of thiophenate and fenbendazole and in the sub-bacillary layer of parasites incubated in 10 μg/ml of thiophenate. A slightly decreased activity was recorded in the epithelium of thiophenate- and fenbendazole-treated worms, and a considerably decreased activity was observed in the sub-bacillary layer of parasites incubat-

Plate I

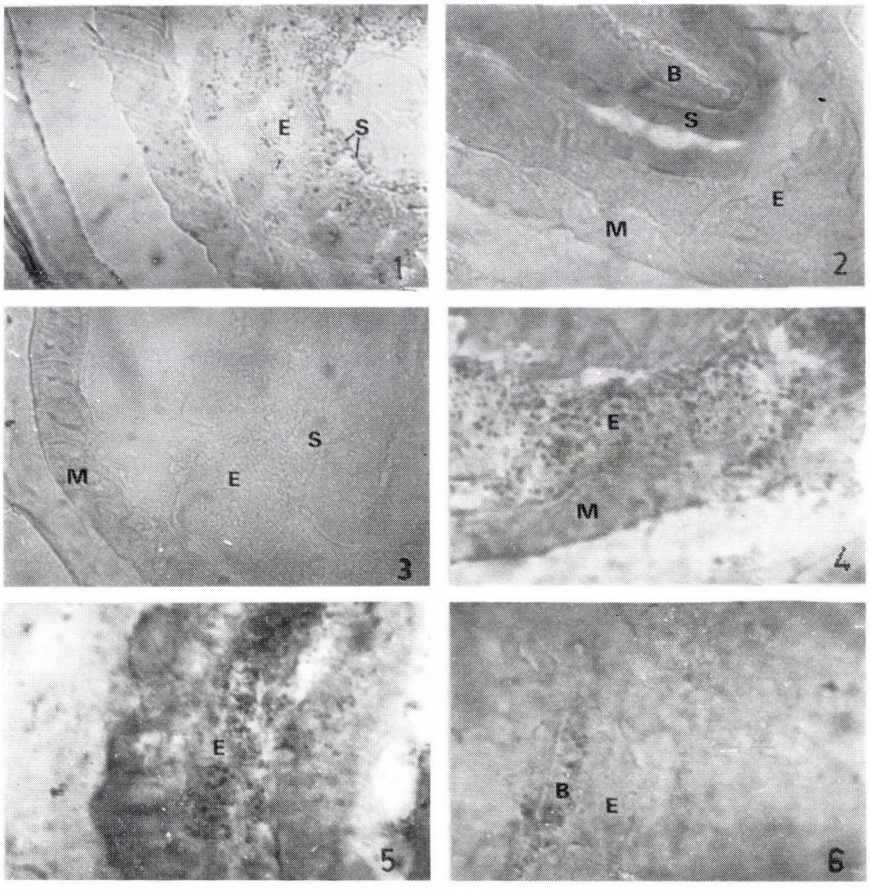


Fig. 1. AkPase activity in thiophenate-incubated (50 μg/ml) *Haemonchus contortus* showing decreased activity in the sub-bacillary (S) layer and epithelium (E). Azo dye method, $\times 500$

Fig. 2. AcPase activity in thiophenate-incubated (50 μg/ml) *H. contortus* showing complete loss of reaction in the muscles (M) and epithelium (E), and weak activity in the sub-bacillary layer (S) and brush border (B). Azo dye method, $\times 500$

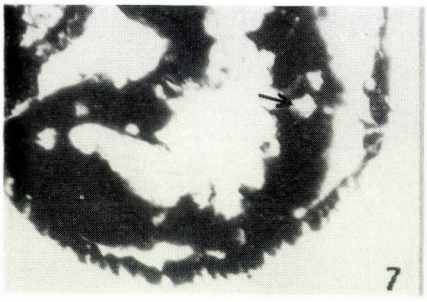
Fig. 3. AcPase activity in fenbendazole-treated (50 μg/ml) treated *H. contortus*. Note the complete loss of activity from the muscles (M), epithelium (E) and sub-bacillary layer (S). Azo dye method, $\times 500$

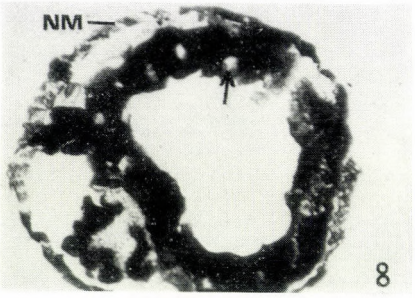
Fig. 4. ATPase activity in thiophenate-treated (10 μg/ml) *H. contortus* showing decreased activity in the muscles (M) and reduced number of ATPase-reactive vacuoles in the epithelium (E). Calcium-activated ATPase method, $\times 1{,}000$

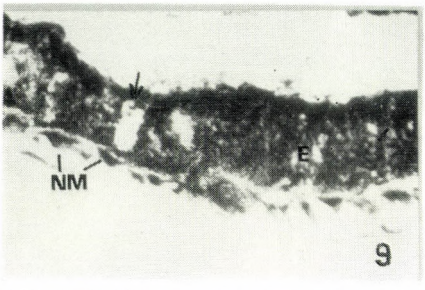
Fig. 5. ATPase activity in fenbendazole-incubated (50 μg/ml) *H. contortus* showing a further decrease in the number of ATPase-reactive vacuoles in the epithelium (E). Calcium-activated ATPase method, $\times 1{,}000$

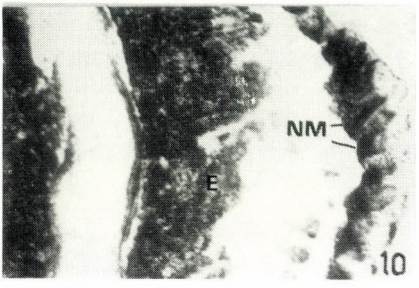
Fig. 6. G-6-Pase in thiophenate-treated (10 μg/ml) *H. contortus* showing loss of reaction from the epithelium (E). Note doubtful to weak reaction in the brush border (B). Lead nitrate method, $\times 800$

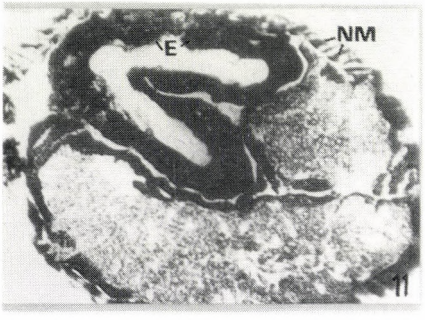
Plate II

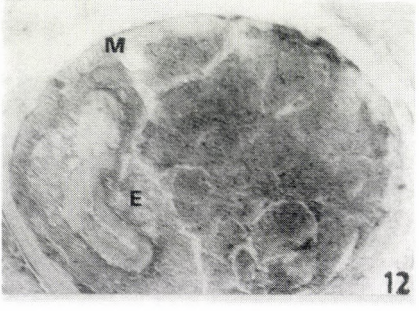


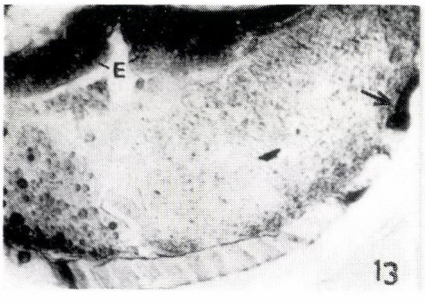












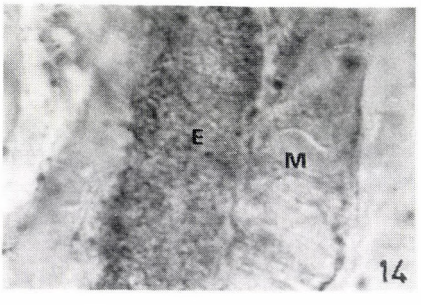
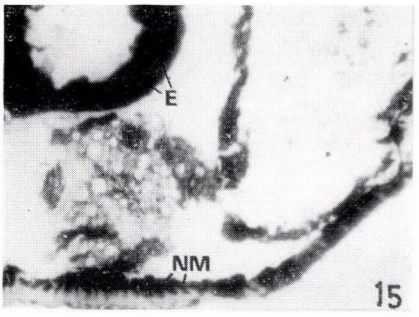
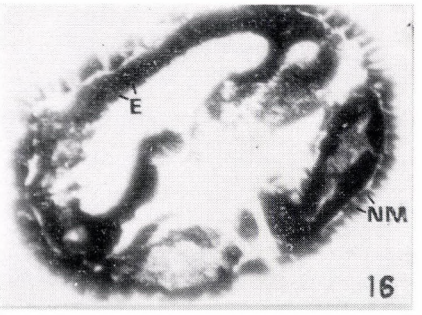
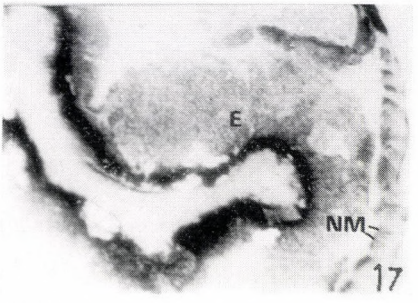
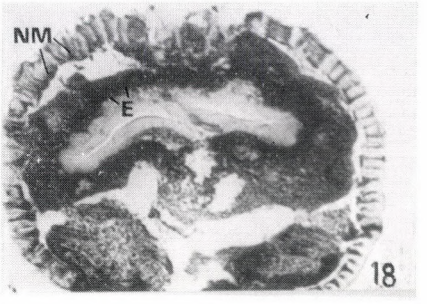


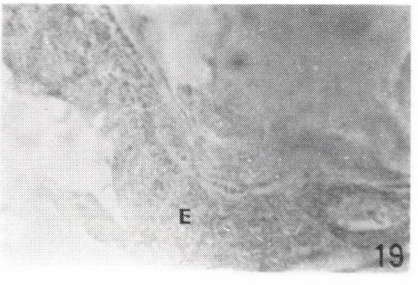
Plate III

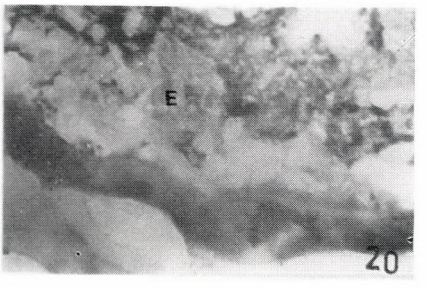


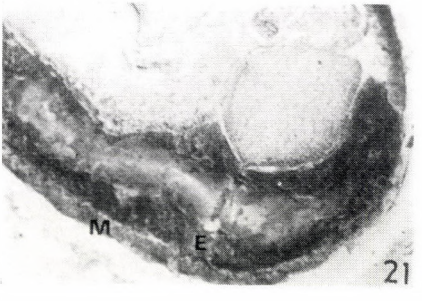












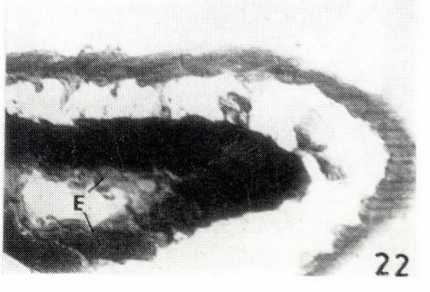


Fig. 7. SDH activity in thiophenate-incubated (10 μg/ml) *H. contortus*. Note large vacuoles (arrow) in the epithelium. Standard method for bound enzymes, ×250

Fig. 8. SDH in fenbendazole-treated (50 μg/ml) *H. contortus* showing moderate activity in the non-contractile part of muscle (NM) and large vacuoles (arrow) in the epithelium. Standard method for bound enzymes, ×250

Fig. 9. LDH activity in thiophenate-incubated (10 μg/ml) *H. contortus*. Note very strong activity in the non-contractile part of muscles (NM) and epithelium (E). Also, observe two large vacuoles (arrow) in the epithelium. Standard method for bound enzymes, ×500

Fig. 10. LDH activity in fenbendazole-treated (50 μg/ml) *H. contortus* showing very strong activity in the non-contractile part of the muscles (NM) and epithelium (E). Standard method for bound enzymes, ×500

Fig. 11. GDH activity in thiophenate-treated (10 μg/ml) *H. contortus* showing very strong activity in the non-contractile part of the muscles (NM) and epithelium (E). Standard method for bound enzymes, ×200

Fig. 12. GDH activity in fenbendazole-treated (10 μg/ml) *H. contortus*. Note complete loss of activity from the muscles (M) and only doubtful reaction in the epithelium (E). Standard method for bound enzymes, ×200

Fig. 13. G-6-PD activity in thiophenate-treated (50 μg/ml) *H. contortus*. Note strong activity in the epithelium (E) and muscles lying underneath the oval body (arrow). Standard method for bound enzymes, ×250

Fig. 14. G-6-PD activity of fenbendazole-treated (10 μg/ml) *H. contortus*, showing loss of activity from the muscles (M) and only weak reaction in the epithelium (E). Standard method for bound enzymes, ×1,000

Fig. 15. NADH-D activity in thiophenate-incubated (50 μg/ml) *H. contortus* showing very strong activity in the non-contractile part of the muscles (NM) and epithelium (E). Standard method for bound enzymes, ×250

Fig. 16. NADH-D activity in fenbendazole-treated (10 μg/ml) *H. contortus* showing strong reaction in the non-contractile part of the muscles (NM) and epithelium (E). Standard method for bound enzymes, ×250

Fig. 17. NADPH-D activity in thiophenate-treated (10 μg/ml) *H. contortus* showing weak activity in the non-contractile part of muscles (NM) and moderate reaction in the epithelium (E), located closer to the luminal border. Standard method for bound enzymes, ×250

Fig. 18. NADPH-D activity in fenbendazole-treated (50 μg/ml) *H. contortus*. Note strong activity in the non-contractile part of the muscles (NM) and epithelium (E). Standard method for bound enzymes, ×250

Fig. 19. CYO activity in thiophenate-treated (50 μg/ml) *H. contortus* showing coarse granular reaction in the epithelium (E). Butcher's method, ×800

Fig. 20. AChEase activity in thiophenate-treated (10 μg/ml) *H. contortus* showing reaction in the epithelium (E). Karnovsky and Roots method, ×500

Fig. 21. NSE activity in thiophenate-treated (10 μg/ml) *H. contortus* showing moderate activity in the muscles (M) and epithelium (E). Indoxyl acetate method, ×200

Fig. 22. NSE activity in thiophenate-treated (50 μg/ml) *H. contortus*. Note strong activtiy in the epithelium (E). Indoxyl acetate method, ×200

ed in both drugs (Fig. 1). After treatment with 10 μg/ml thiophenate, the granules were more concentrated near the sub-bacillary layer, whereas the epithelium contained some vacuoles.

A complete loss of AcPase activity was recorded in the muscles, while a greatly reduced reaction was observed in the sub-bacillary layer and brush borders of intestine of the parasites treated with either drug (Figs 2 and 3). After treatment with 10 μg/ml fenbendazole, the epithelium exhibited a weak reaction, located closer to the brush border side, whereas 50 μg/ml of the same drug caused a complete loss of AcPase from all the layers (Fig. 3).

The epithelium of non-incubated and Tyrode-incubated worms exhibited very large vacuoles with ATPase-positive boundaries which were located closer to the brush border side of the intestine. Only a few small vacuoles were observed in the epithelium of parasites treated with 10 and 50 μg/ml thiophenate (Fig. 3) and 10 μg/ml fenbendazole. The number of vacuoles further decreased after treatment with 50 μg/ml fenbendazole (Fig. 4). Decreased ATPase activity was observed in the contractile part of the muscles of thiophenate- and fenbendazole-treated (Fig. 5) parasites, whereas a slightin crease of such activity was recorded in the hypodermis of fenbendazole-incubated worms.

Thiophenate caused the complete loss of reactive vacuoles of G-6-Pase from the hypodermis, muscles, epithelium and sub-bacillary layer, the brush borders being only doubtful to weakly reactive (Fig. 6). The effects of fenbendazole were even more drastic, leading to the complete loss of enzymic reaction of G-6-Pase from all layers of the parasites.

Dehydrogenases (Plate II). SDH decreased to a moderate reaction in the hypodermis of thiophenate- and fenbendazole-incubated parasites and in the non-contractile part of muscles of fenbendazole-treated worms (Figs 7 and 8). SDH reaction was strong in the epithelium and sub-bacillary layers. Very large vacuoles were observed in the epithelium of thiophenate- and fenbendazole-incubated worms.

LDH exhibited a highly increased activity in the non-contractile part of muscles, epithelium and sub-bacillary layers of parasites incubated in either of the two drugs (Figs 9 and 10). A strong LDH activity was observed in the hypodermis of thiophenate- and fenbendazole-treated parasites. In the brush borders of thiophenate-incubated worms, a weak activity was observed in some places, whereas at other locations LDH activity was nil. Very large vacuoles were observed in the epithelium of thiophenate-treated parasites, the reaction being stronger towards the luminal side (Fig. 9). The reaction took the form of coarse granules in the fenbendazole-treated parasites. In this case also, the reaction was located closer to the luminal side (Fig. 10).

The worms showed increased GDH activity in the epithelium, non-contractile part of the muscles and hypodermis of the thiophenate-treated parasites (Fig. 11), while a marked decrease in activity was recorded in the

hypodermis and epithelium and a complete loss of activity in the non-contractile part and sub-bacillary layer of fenbendazole-incubated worms (Fig. 12).

As compared to the Tyrode-treated worms, thiophenate-treated parasites displayed an increased amount of G-6-PD in the hypodermis and epithelial layer (Fig. 13). Muscles lying underneath the oval bodies were strongly positive. A complete loss of G-6-PD activity was observed in all the layers except the epithelium where the reaction was diffuse in the fenbendazole-incubated parasites (Fig. 14).

Diaphorases (Plate III). A slightly increased activity was recorded in the epithelium of thiophenate- and fenbendazole-treated parasites (Figs 15 and 16), while a slightly decreased activity was recorded in the hypodermis of parasites treated with either drug.

As compared to the Tyrode-incubated parasites, NADPH-D activity was reduced to moderate in the epithelium of the thiophenate-treated parasites, whereas a weak reaction was observed in the non-contractile part of the muscles (Fig. 17). The epithelium showed strong but diffuse activity in the fenbendazole-treated parasites (Fig. 18) and the reaction was located closer to the luminal side.

Oxidases (Plate III). CYO activity in parasites treated with either drug was similar to that of the Tyrode-incubated parasites. Coarse granular activity was observed in the epithelium and the reaction was of diffuse type in the brush borders of the intestine (Fig. 19).

MAO also showed the same activity as that of Tyrode-treated parasites in all layers of worms incubated in thiophenate and fenbendazole.

Esterases (Plate III). The epithelium contained very large AChEase-reactive vacuoles which remained unaffected after drug treatment (Fig. 20). A slightly increased MSE activity was observed in the epithelium and contractile part of muscles in parasites treated with 10 μg/ml thiophenate (Fig. 21). The activity increased to high intensity in the epithelium after treatment with 50 μg/ml of the same drug (Fig. 22). There was no change in the enzyme activity patterns of fenbendazole-incubated parasites.

Discussion

From the results of the present study it appears that the enzymatic intensities and the alterations induced in *H. contortus* by drugs are stronger in the intestine than in the body wall. This is in contrast to the findings of Hobson et al. (1952*a*, *b*), who found the cuticle of some ascarids permissible for inorganic ions. Our conclusion is similar to that derived by Pappas and Read (1975).

The decreased activity of AkPase after treatment with thiophenate and fenbendazole is indicative of an impairment of the transmembrane active transport of metabolites. The effect of these drugs is, however, in contrast to that exerted by rafoxanide on *H. contortus* (Kaur and Sood, 1990*a*). Kaur and Sood (1990*a*, *b*) found similar AkPase activities after treating the parasites with nilzan, thiabendazole, nilverm and nilverm forte.

The effect of these two drugs on AcPase was more drastic than their influence on AkPase activity. This enzyme has also been shown to be inhibited by dl-tetramisole (Chakraborty et al., 1976; Kaur and Sood, 1982*a*). The present findings suggest that the intracellular digestion of drugs may involve lysosomes (Colam, 1971). Chopta and Premvati (1982) also concluded that AcPase plays a role in the phosphorylation mechanisms of carbohyd rate metabolism of adult *H. contortus*.

Adenosine triphosphatase detected in adult *H. contortus* is involved in the absorption of nutrients and active transport (Kaur and Sood, 1982*c*). This enzyme is also associated with muscle contraction (Jenkins, 1970). A decreased activity in the contractile part of muscles after treatment with either drug is suggestive of an impairment of muscle contraction. These findings are in agreement with those of Kaur and Sood (1990*a*, *b*), where a decreased activity was found in the contractile part of muscles.

The impaired activity of G-6-Pase in all the layers after thiophenate and fenbendazole treatment may be related to the inhibiton of glucose uptake and transport. Similarly, dithiazine (Bueding et al., 1966), thiabendazole, morantel tartrate, tetramisole hydrochloride (Sood and Kaur, 1982), nilzan (Kaur and Sood (1990*a*), nilverm and nilverm forte (Kaur and Sood, 1990*b*) also caused the inhibition of this enzyme in the intestine of *H. contortus* and other helminths. However, rafoxanide (Kaur and Sood, 1982*a*) increased the activity of this enzyme.

SDH activity remained unaffected except in the epithelium and subbacillary layers of the intestine of thiophenate- and fenbendazole-incubated parasites, where it increased. SDH is an enzyme of the TCA cycle which has a minor function in the energy metabolism of the parasite (Kaur and Sood, 1983). Our findings are in contrast to those of Comley and Wright (1981) and Kaur and Sood (1990*a*, *b*), who found this enzyme to be inhibited by various drugs.

LDH catalyses the conversion of pyruvate to lactate under anaerobiasis and is present in several nematodes (Anwar et al., 1977; Kaur and Sood, 1982*b*; Walter and Schulz, 1980). The afore-cited findings indicate that the anaerobic pathway of CO_2 production from glucose is considerably stimulated by the drugs as also reported by Ward (1974). Our findings are in contrast to those of Doong et al. (1987), who showed LDH activity to be inhibited by hycanthone in drug-sensitive strains of *Schistosoma mansoni*. However, the present results

are in agreement with our earlier findings (Kaur and Sood, 1990*a*, *b*) in that activity shows an increase in all the positive layers.

GDH showed increased activity in the thiophenate-treated parasites. This is similar to that detected by us earlier (Kaur and Sood, 1990*a*) after treating the parasites with nilzan and albendazole. However, GDH activity showed a decrease in the fenbendazole-treated worms. The enzyme plays a central role in amino acid deamination and in the fixation of the α-aminonitrogen group from ammonia (Kapur et al., 1984).

The higher activity of G-6-PD in the hypodermis and epithelium of thiophenate-treated parasites is suggestive of a stimulation of the pentose phosphate pathway. The enzyme has been shown to be related to requirements of large amounts of ribose or reducing powers (NADPH) required for biosynthetic purposes (Kapur and Sood, 1986; Korting and Barrett, 1977). Biochemically, there is no evidence that the pathway has any relation with the energy metabolism in nematodes (Croll, 1976). The present findings are similar to our earlier results (Kaur and Sood, 1990*a*, *b*). This enzyme has been shown to be decreased in all the layers except the epithelium where the reaction was of diffuse type. These observations fully agree with the results of Pompori and Srivastava (1987) who found the enzyme to be inhibited in intact *Cotugnia digonopora*.

NADH-D activity showed a slight increase in the epithelium of thiophenate- and fenbendazole-treated parasites, while the enzyme remained unaltered by albendazole treatment (Kaur and Sood, 1990*a*), and a marked decrease of activity was observed in the intestine after treatment with 50 μg/ml of nilzan.

NADPH-D activity was shown to be reduced to moderate intensity in the epithelium of thiophenate-treated parasites. Fenbendazole-incubated parasites showed a diffuse activity in the epithelium. This is in contrast to our earlier results (Kaur and Sood, 1990*a*) according to which this enzyme was unaltered by treatment with albendazole.

Anthelmintics such as nilverm and nilverm forte (Kaur and Sood, 1990*b*) have been demonstrated to inhibit a decrease in the CYO as well as MAO activity in various layers of the parasite. However, the anthelmintics tested in the present study, thiophenate and fenbendazole, did not cause any appreciable change in the activity of these oxidases.

Only the 50 μg/ml dose of thiophenate could stimulate NSE in the gut epithelium. However, fenbendazole did not induce any alterations in their distribution pattern. Earlier findings on *H. contortus* showed that albendazole (Kaur and Sood, 1990*a*) and nilverm forte (Kaur and Sood, 1990*b*) also alter the distribution of NSE.

Acknowledgements

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DEMONSTRATION OF *CLOSTRIDIUM SEPTICUM* INFECTION IN A GOOSE FLOCK

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Clostridium septicum infection causing 5.0 to 5.2% mortality is reported for the first time in the literature from six-week-old growing geese in three flocks comprising 5,200, 5,500 and 5,900 geese, respectively. The affected birds exhibited weakness, incoordinated movement, ataxia and, frequently, oblique position of the head and neck (torticollis) as well as signs indicative of dysequilibrium. The affected birds died within 18–24 h.

Gross pathological examination revealed anaemia, hepatitis with map-like necroses of irregular outline (Fig. 1), acute enteritis, pulmonary oedema and cardiac dilatation. Light and electron-microscopic examination showed that the sinusoids of the liver were markedly dilated (Fig. 2) and filled with serous exudate and gas (Figs 2 and 3), and the hepatocytes surrounding them exhibited severe oedema (Fig. 4). Among the hepatocytes, ciliated bacteria 7–10 μ in length and 1–3 μ in width, bounded by a well-defined cell wall and often showing signs of spore formation were observed (Figs 5 and 6).

By bacteriological examination the pathogen was isolated, its properties were studied, and the clinical entity of malignant oedema was experimentally reproduced by intramuscular injection of guinea-pigs and rabbits.

The applied antibiotic (oxytetracycline) and furazolidone therapy proved effective.

Key words: *Clostridium septicum*, infection, goose, mortality, clinical signs, pathology, first report

Clostridium septicum is known to be one of the causative agents of malignant oedema in mammals. In poultry species it rarely causes disease: so far it has been reported to do so only in chickens and turkeys (Hofstad et al., 1988).

Saunders and Bickford (1965) and Helfer et al. (1969) observed disease cases and deaths due to *Cl. septicum* in flocks of 6–12 weeks old broilers. Morbidity and mortality ranged between 5 and 60%. The clinical signs included anorexia, depression, incoordinated movement and ataxia. Death usually occurred 18–24 h after the onset of the clinical signs. At necropsy, serosanguineous infiltration and gas bubbles were observed in the skin of the legs and ventral wall of the abdomen, in the subcutaneous connective tissue and the adjacent muscle tissue. Areas showing skin lesions and circumscribed feather loss, with no pathological lesions in the inner organs, were also common. These sites may have served as port of entry for the pathogen. *Cl. septicum* was microscopically demonstrable in, and could be cultured from, the

serosanguineous exudate. Guinea-pigs and broiler chickens intramuscularly inoculated with the isolate developed lesions typical of *Cl. septicum* and died within 14–24 h.

Hofacre et al. (1986) observed a similar disease in broiler flocks, too. Besides serosanguineous infiltration of the subcutaneous connective tissue and musculature of the abdominal wall and the thighs, severe enteritis and inflammation of the bursa of Fabricius, characteristic of infectious bursal disease, were also seen at necropsy. At the same time, no outward signs of injury were seen on the skin surface. The authors suggested that clostridia may have entered the organism through the intestinal wall as a result of the birds' impaired general resistance and enteritis.

In the case presented here, *Cl. septicum* infection was diagnosed in a goose flock. As no description of such a case is available in the literature either in Hungary or abroad, we felt that our findings would be worth reporting.

Materials and methods

Case history

In three large goose flocks comprising 5,200, 5,500 and 5,900 geese, respectively, a disease characterized by weakness, incoordinated movement, ataxia and, often, other nervous signs (oblique position of the head, torticollis, dysequilibrium) was observed among the 6 weeks (39–43 days) old growing birds over a period of 13–15 days. The affected birds usually died in one day's time. The mortality rate was about 5.0–5.2%.

The affected birds often exhibited severe diarrhoea and excreted blood-stained faeces.

The geese were kept in a half-open goose shed having a large, sloping, sandy-soiled outdoor run. They were fed an own-made starter then grower diet. Water pumped out of a brook, disinfected with chloride of lime and collected in concrete tanks was used as drinking water.

Gross and histopathological examination

Gross pathology. Birds that had died of the infection were found retarded in growth and markedly anaemic at necropsy. Body parts not covered by feathers, mainly the beak and the feet, were pale yellow or orange-coloured instead of the normal brick-red colour. The conjunctiva and the visible mucous membranes were also pale. The liver was swollen and had small, grayish-white, sometimes confluent foci of irregular shape or large necrotic areas of map-like outline on both its surface and cut surface (Fig. 1). From the pathologically changed areas a serous exudate containing gas bubbles could be squeezed

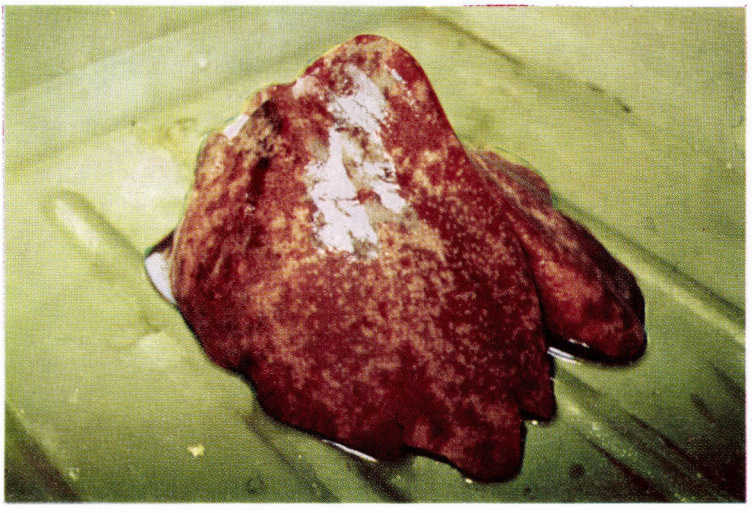

Fig. 1. Grayish-white, here and there confluent necroses of irregular shape in the liver

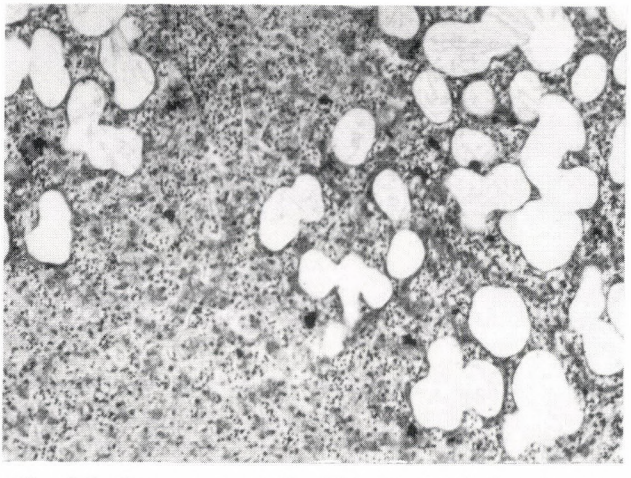

Fig. 2. The sinusoids of the liver are markedly dilated and only in a few places is there a little content in their lumen. Haematoxylin and eosin (H.–E.), ×63

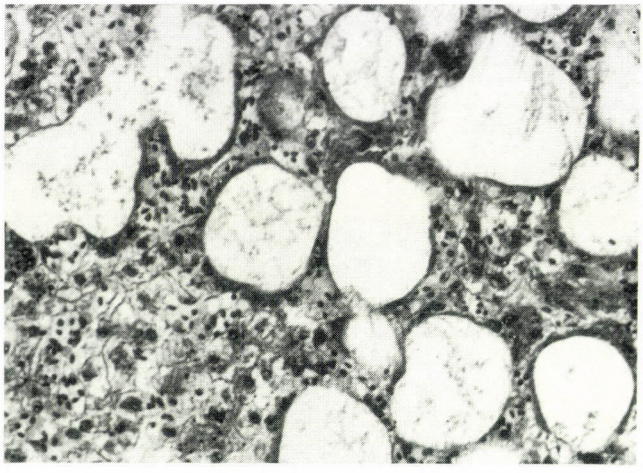

Fig. 3. The sinusoids of the liver are markedly dilated and only in a few places is there a little content in their lumen. Haematoxylin and eosin (H.–E.), ×160

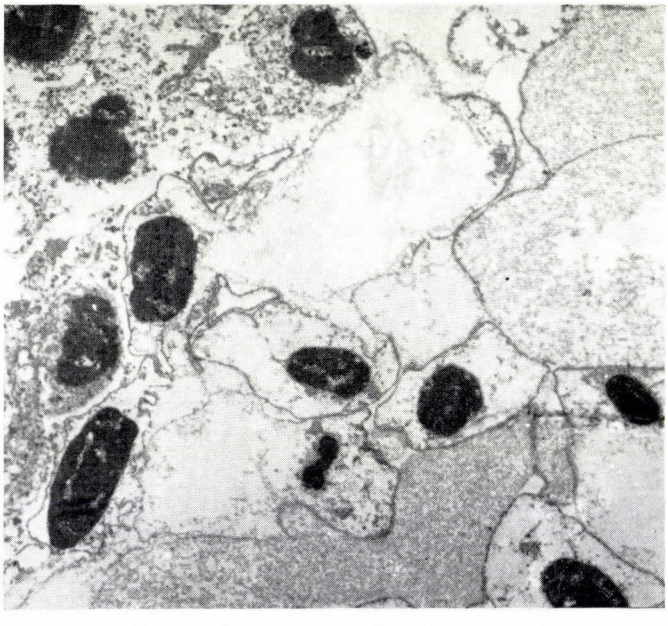

Fig. 4. Oedema in the cytoplasm of hepatocytes and in the intercellular spaces, with lysis of the cell organelles and karyopycnosis. Electron micrograph, ×8,000

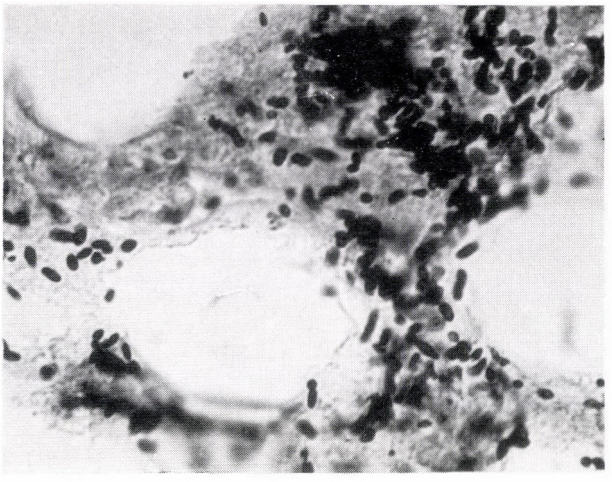

Fig. 5. Masses of rod-shaped bacteria among the hepatocytes. Gram staining, ×400

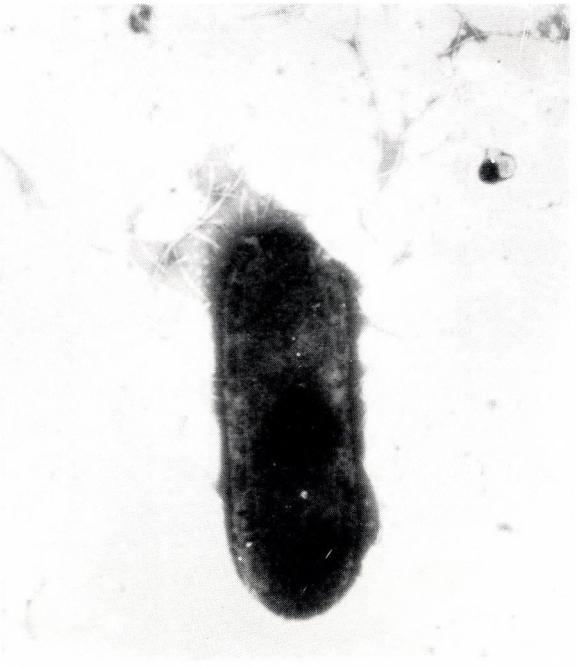

Fig. 6. Cilia on the thick cell wall of the bacteria. Electron micrograph, negative contrast preparation, ×29,200

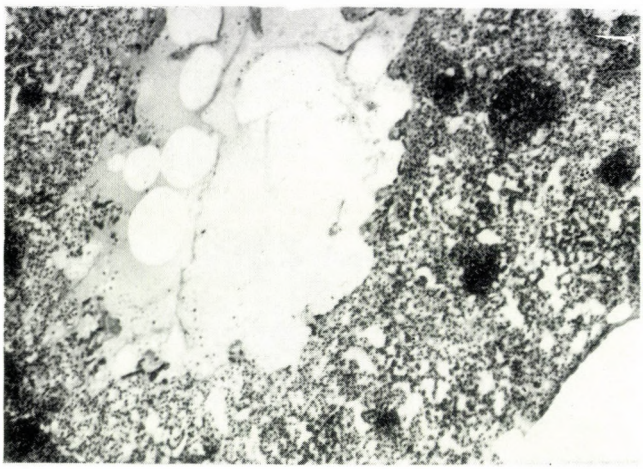

Fig. 7. Accumulation of serous exudate in the air passages of the lungs. H.–E., ×6.

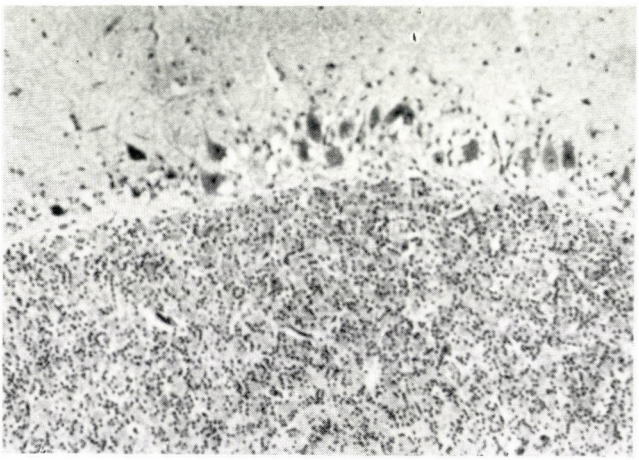

Fig. 8. Serous exudation in the Purkinje's ganglion cell layer of the cerebellum. H.–E., ×63

out. The intestinal mucosa was rose-red, swollen and had catarrhal mucus on its surface. In the cavity of the gizzard grains of sand could be recognized. The lungs were hyperaemic and their cut surface was very succulent. The heart was dilated, the myocardium was pale and easy to tear, and in the pericardium a serous exudate had accumulated. The kidneys were swollen and hyperaemic. The spleen was normal. Neither the subcutaneous connective tissue nor the muscles showed any pathological changes.

Light and electron microscopic histopathological examination. The sinusoids of the liver were markedly dilated in some places (Fig. 2) and serous exudate and gas had accumulated in their lumen (Fig. 3). Accumulation of serous exudate and gas in the cytoplasm of the neighbouring hepatocytes had led to structural disintegration of those cells, lysis of most of their cell organelles, and karyopycnosis (Fig. 4). Large numbers of rod-shaped bacteria 7–10 μ in length and 1–2 μ in width, bounded by a well-defined cell wall and occasionally showing signs of spore formation, were seen in the intercellular spaces (Fig. 5). The bacteria had cilia (Fig. 6) on their cell wall. In the hyperaemic lungs, serous exudate had accumulated in the lumen of some of the air passages (Fig. 7). Signs of serous exudation were observed also in the Purkinje's ganglion cell layer of the cerebellum (Fig. 8).

Bacteriological examination and inoculation of experimental animals

The pathogen was successfully isolated from the liver under anaerobic conditions at 37 °C both in Tarozzi's liver broth with gas formation and on blood agar. It lysed red blood cells on blood agar, showed motility and split glucose, maltose and lactose but not saccharose and mannitol in semi-solid medium. The bacterium was urease, indole, lecithinase and lipase negative but positive for aesculin as well as gelatin hydrolysis.

Guinea-pigs and rabbits intramuscularly inoculated with the homogenate of pathologically altered goose liver and its 24-h cultures in Tarozzi's liver broth died within 24 h. At the site of injection the muscles showed serosanguineous infiltration and contained gas bubbles. The pathogen was demonstrable in impression smears made from the peritoneum and muscles showing pathological lesions.

Medication

On the basis of its morphological as well as biochemical properties and its pathogenicity, the isolate was identified as *Cl. septicum*. *In vitro* the isolate was sensitive to penicillin, erythromycin, Tetran, furazolidone, Linco-Spectin and Imequil, moderately sensitive to Sumetrolim, and resistant to neomycin, oleandomycin and polymyxin B.

Treatment with Erra-6 (an oxytetracycline-containing medicated premix) and furazolidone 3% medicated premix prevented further disease cases and deaths in a few days.

Discussion

In the case presented in this paper, *Cl. septicum* caused morbidity and mortality in a goose flock. No such data have been reported in the special literature either in Hungary or abroad. In earlier cases reported from broiler flocks (Saunders et al., 1965; Helfer et al., 1969), *Cl. septicum* caused a disease entity manifesting itself as gangrenous dermatitis in which the probable ports of entry were skin injuries. Hofacre et al. (1986) also reported gangrenous dermatitis from broiler chickens infected by *Cl. septicum;* however, they did not observe skin injuries and assumed that the pathogen's port of entry must have been the intestinal mucosa the resistance of which had been impaired by inflammation. In our case, too, the pathogens may have entered the organism through the inflammatory intestinal mucosa. Namely, the birds examined by us had no lesions in the skin, subcutaneous connective tissue and musculature, and the pathological picture was dominated by hepatitis accompanied by oedema, gas formation and necrosis. Also, it was the liver from which the pathogenic bacteria could be isolated. Clinical manifestation of the disease may have been facilitated by the impaired general resistance of the flock which had been affected by Derzsy's disease at 2–3 weeks of age and by salmonellosis at 4–5 weeks of age.

The applied antibiotic (oxytetracycline) and furazolidone treatment proved effective.

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STUDIES ON CANINE MAMMARY TUMOURS I. AGE, SEASONAL AND BREED DISTRIBUTION

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The incidence as well as age, seasonal and breed distribution of canine mammary tumours (n = 521) were studied at the Clinic of Obstetrics and Gynaecology of the University of Veterinary Science, Budapest, between 1985 and 1989. In 39 cases of mammary tumour, blood plasma oestradiol (E_2) and progesterone (P) concentrations were also determined. Of all dogs referred to the clinics of the University in 1985, 0.7% had mammary tumour. On the average, 104 $\pm$ 9.3 cases of mammary tumour were recorded at the Clinic of Obstetrics per year. This number did not increase after the Chernobyl atomic reactor catastrophe of 1986. The age distribution of canine mammary tumour found in this study shows good agreement with earlier data of the literature: mammary tumour showed the highest incidence in 10 years old dogs. The incidence of mammary tumour kept increasing with age until the 14th year of life (as expressed in per cent of animals of identical age). The number of mammary tumours was markedly higher in the spring (April–May) and autumn (September). This seasonality was demonstrable in 11 to 16 years old bitches, too. On the basis of the blood plasma E_2 and P profiles, 61.5% of the clinically anoestrous animals were found to be cycling. The strikingly high ratio of pulis among dogs with mammary cancer was suggestive of a breed disposition.

Key words: Canine mammary tumour, dog, seasonal distribution, age, breed disposition

The high incidence of canine mammary tumours in the veterinary practice should certainly deserve more attention than it has received so far. B. Kovács and Somogyvári (1968) analyzed the statistical data available on the occurrence of neoplasms in domestic animals hospitalized at the Clinic of Surgery, University of Veterinary Science, Budapest, between 1914 and 1964. The overall incidence of tumors in the patients examined was 4.5%; within this, the incidence of tumours in dogs was found to be 9.1%. The incidence of mammary tumours in dogs at the same clinic during the subsequent 10 years was reported by Szokolóczy (1974). We found that 9.5% of the dogs treated at the Clinic of Obstetrics in 1985 had some kind of tumour.

Our data show that in the 1980's the incidence of canine tumours in dogs treated at the Outpatient Clinic of the University of Veterinary Science was around 0.4%, which is almost twice that reported from the United States (Hamilton et al., 1977). It is interesting that the incidence of human tumours in Hungary is also twice that occurring in the U. S. A. (Eckhardt, 1977). The special literature unanimously states that mammary tumour is the commonest neoplastic disease of the dog (Moulton et al., 1970).

In Hungary, Csenki and Tory (1983) were the first who attempted hormone therapy of mammary tumours in elderly dogs. Although tumour cell steroid hormone receptor and plasma steroid level determinations were already known at that time, no veterinary study has yet been undertaken to identify factors that may influence the success of using hormone preparations for the therapy of mammary tumours.

The significance of studies on canine mammary tumour is further increased by its suitability for use as a model in studying the aetiology, pathogenesis and therapy of human mammary carcinoma. This is rendered possible by similarities in biological properties, spontaneous occurrence, age and hormonal dependence, pathogenesis and, last but not least, sexual steroid characteristics, as well as parallelisms regarding the use of oral contraceptives. However, there are significant differences between the mammary tumours of dogs and humans. These differences may be due primarily to the fact that dogs usually come into oestrus twice a year while women have 28-day cycles.

In this work, previously not studied aspects of canine mammary tumour incidence and its distribution by age, season and breed were investigated, with special respect to the stage of cycle determined from the sexual steroid hormone concentrations of the blood plasma.

Materials and methods

The patient material used in this study was constituted by all tumourous dogs treated at the Clinic of Obstetrics between 1985 and 1989 either as outdoor or as hospitalized patients (n = 521). The age of the animals was between 4 and 13 years. Furthermore, we compared the number of all canine patients (n = 26,202) treated at the clinics of the University of Veterinary Science in 1985 with that of dogs affected with mammary tumours (n = 188) in the same year.

Results of the histological examination of mammary tissue samples are reported in the accompanying paper (Boldizsár et al., 1992). In 39 cases, the 17ß-oestradiol (E_2) and progesterone (P) concentrations of the blood plasma were also determined by radioimmunoassay (Abraham et al., 1971; Mikhail et al., 1970) at the time of the operation, in most cases at 9 a. m. P was extracted with petroleum ether while E_2 with diethyl ether. The radioactivity of samples labelled with ^3H was measured by a liquid scintillation method.

On the basis of the 17ß-oestradiol and progesterone concentrations of the blood plasma, the dogs were assigned to different phases of the cycle. The limit for anoestrus was 18 pg/ml and 10 ng/ml for E_2 and P, respectively.

Results

The data presented in Table 1 show that 0.7% of all dogs treated at the clinics of the University of Veterinary Science in 1985 had mammary tumours.

The breed, age and seasonal (month) distribution of dogs treated for mammary tumour at the Clinic of Obstetrics between 1985 and 1989 was determined. During that period, a total of 521 dogs (104 $\pm$ 9.3/year) were treated for mammary tumour at the clinic. Of them, 348 were pedigree dogs. No significant differences were found in mammary tumour incidence by year. The number of cases was not significantly higher in the year of the Chernobyl nuclear reactor catastrophe (1986): in that year 103 dogs were treated for mammary tumour at the clinic (Table 2). For comparison, the table gives the human mortalities caused by mammary carcinoma in the period of study (Hungarian Annals of Statistics, 1985–1989).

Table 1

The total numbers of canine patients and those of dogs treated for mammary tumour at the clinics of the University of Veterinary Science in 1985

Clinic	Total number of canine patients	Patients with mammary tumour
Clinic of Veterinary Medicine	8,386	14
Clinic of Surgery	7,376	25
Clinic of Obstetrics	1,197	114
Outpatient Clinic	9,243	35
Total:	26,202 (100%)	188 (0.7%)

Table 2

Incidence of canine mammary tumours and mortalities from human breast cancer in the years 1985 through 1989

Species	Years				
	1985	1986	1987	1988	1989
Cases of canine mammary tumour*	114	103	96	94	114
Mortalities from human breast cancer**	1,999	2,015	2,119	2,021	2,112

* at the Clinic of Obstetrics, University of Veterinary Science;
** data from the Hungarian Annals of Statistics (1985–1989)

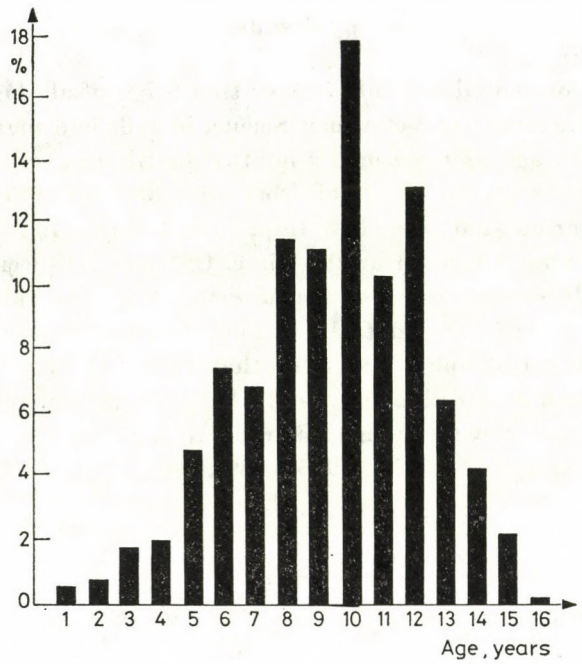

Fig. 1. Age distribution of canine mammary tumours (n = 521) in a 5-year period, in %

The age distribution of canine mammary tumour patients treated at the Clinic of Obstetrics is shown in Fig. 1. The number of cases starts to rise steeply from 8 years of age, reaches the peak at the age of 10 years, and a marked decrease occurs only from 13 years of age onward.

The age distribution found by us was compared with the statistical data of Szokolóczy (1974) relating to canine mammary tumour incidence in a ten-year period and with the data of B. Kovács and Somogyvári (1968) on overall tumour incidence in domestic animals (Fig. 2). Our data show good agreement with those of Szokolóczy (1974) but, as was expected, differ from age distribution data of tumours in *all animals* treated at the university clinics (B. Kovács and Somogyvári, 1968). The mode of the curve describing overall tumour incidence in domestic animals is shifted forwards and tumour incidence at an early age is high.

If the ratio of tumour patients is expressed as per cent of all dogs treated at the Clinic of Obstetrics during a period of 5 years (Fig. 3), it appears that the number of disease cases strikingly rises after the 5th year of life, continues to increase until 14 years of age and only then does it start to decline rapidly.

The seasonal distribution of dogs with suspected mammary tumour (n = = 521) was characteristic. Two peaks were observed: one in the spring, in April (or May) and another in the autumn, in September (Fig. 4). Dogs older than 9 years showed a minor peak of mammary tumour incidence in November.

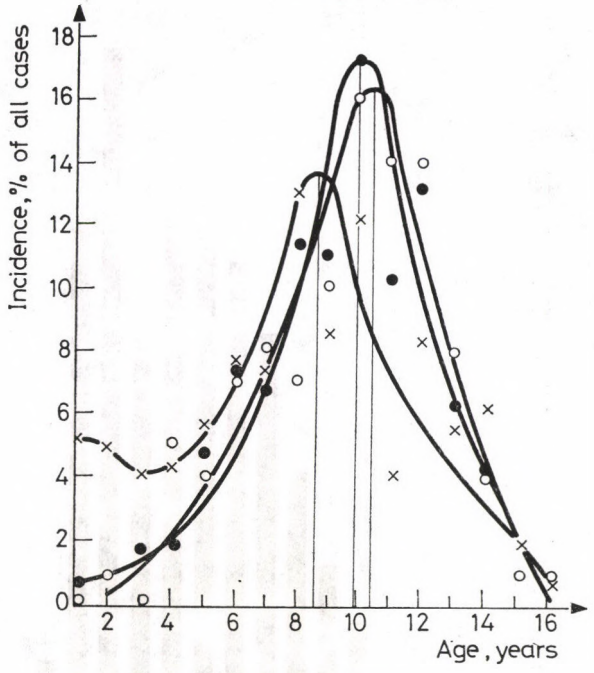

Fig. 2. Tumour incidence (%) in domestic animals according to B. Kovács and Somogyvári (1968) and incidence of canine mammary tumours (%) as found by Szokolóczy (1974) and in our study, as a function of age [● = incidence of canine mammary tumours at the Clinic of Obstetrics in the years 1985 through 1989 (our own results); ○ = incidence of canine mammary tumours at the Clinic of Surgery between 1964 and 1974 (Szokolóczy, 1974); x = overall incidence of tumours in domestic animals at the Clinic of Surgery between 1914 and 1964 (B. Kovács and Somogyvári, 1968)]

Studying the patient material by age, it can be established that the above-mentioned two seasonal peaks in mammary tumour incidence are consistently demonstrable in dogs more than 6 years old (Fig. 5a and 5b). These two peaks were detectable also in 11–16 years old, sexually inactive bitches (Fig. 5c), especially in those aged 14–15 years. The seasonal distribution of canine mammary tumours was studied in 1986, the year of the Chernobyl catastrophe, and in the subsequent years. The seasonal distribution curve obtained for a patient material the average age of which was 10 years did not markedly differ from that found for dogs of similar age in other years.

The breed disribution of dogs treated for mammary tumour at the Clinic of Obstetrics in the period of study is presented in Table 3. The percentile proportion of the different breeds in the patient material and the percentage of dogs pedigreed in Budapest within the individual breeds are also given. (Practically all dogs treated at the Clinic of Obstetrics come from Budapest). When comparing the numbers of tumourous dogs with those of all dogs treated, it is striking to note that certain dogs of large body size (komondor, Newfound-

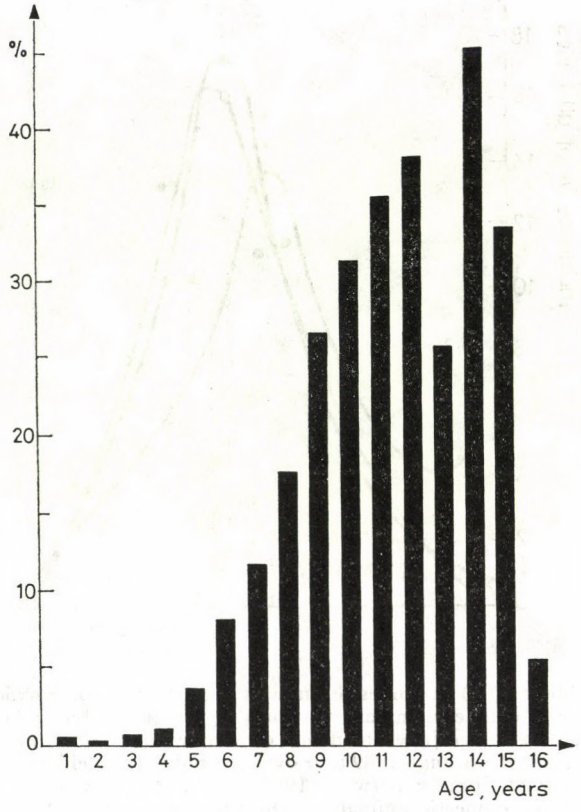

Fig. 3. Incidence of mammary tumour (%) among canine patients treated at the Clinic of Obstetrics (n = 521)

land) and terriers of small or medium body size show significantly (by an order of magnitude) lower incidence of tumours than the puli, a dog of small body size. The following breeds showed a breed disposition for mammary tumour (as evidenced by their higher percentage among dogs with mammary tumour than among all dogs treated), in decreasing order of mammary tumour incidence: puli, setters, poodle, dachshund, fox terrier. These results show that, besides breeds reported to be disposed to mammary tumour (Heinen, 1976), the puli is also highly susceptible to the disease.

In 39 dogs randomly selected from among the 521 patients (average age: 9 (4–13) years), blood plasma 17ß-oestradiol and progesterone concentrations were determined at the time of the surgical removal of mammary tumour.

At the time of the operation, the E_2 concentration of the blood plasma ranged between 1.1 and 84.4 pg/ml and its P concentration was between 1.5 and 36.7 ng/ml. The majority of the dogs seemed to be anoestrous at the time of the clinical examination. Blood plasma sexual steroid hormone concentra-

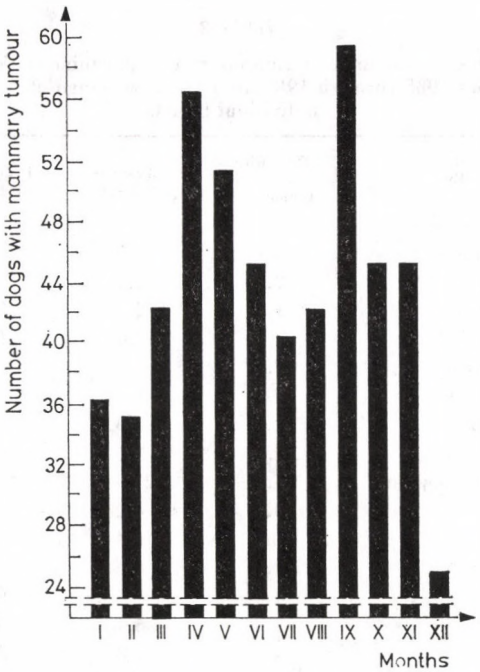

Fig. 4. Seasonal distribution of canine mammary tumours (n = 521) at different ages in the years 1985 through 1989

tions, however, indicated that only 15 dogs (35%) were actually anoestrous; the reamining animals could be assigned to different stages of the cycle (6 dogs: prooestrus; 8 dogs: oestrus; 10 dogs: metoestrus) on the basis of the known hormonal relationships (Table 4).

Discussion

The evaluation of veterinary statistics concerning dogs with mammary tumour is hampered by several factors: e.g. the course of disease is almost impossible to monitor, the files and pedigree records are often incomplete, the identification of breeds is difficult as their names vary by country, the birth and mortality records are unreliable, the living conditions of bastard animals are highly varied, etc.

In the past, a typical shortcoming of veterinary clinical statistics was that they often failed to classify tumours by their stage (Misdorp and Hart, 1990). Due to this deficiency, the mortality rate caused in carnivores by neoplastic diseases cannot be determined accurately. These shortcomings of the statistics do not alter the fact that works dealing with this subject deserve

Table 3

Breed distribution of dogs with mammary tumour and of all canine patients treated at the Clinic of Obstetrics in the years 1985 through 1989, and the percentage of pedigreed dogs within the individual breeds*

Breed	Dogs with mammary tumour, %	All canine patients, %	Pedigreed dogs, %
Greyhound	0.8	0.9	6.6
Boxer	2.3	2.4	1.6
Dobermann	1.8	2.9	5.8
Dog	1.5	2.6	3.7
Fox terriers	2.5	2.1	2.1
Komondor	0.3	1.5	2.1
Kuvasz	2.5	2.9	14.0
Foxhound	1.0	3.7	0.8
German shepherd	31.2	27.5	14.1
Puli	11.3	4.2	5.1
Pointer	0.3	0.4	6.7
Pinscher	1.8	6.6	5.7
Schnauzer	1.5	4.4	6.8
Spaniel	4.8	5.4	6.6
Setters	4.0	2.8	3.2
Dachshund	19.1	15.5	3.3
Terrier	0.5	2.5	4.2
Newfoundland	0.3	1.5	2.4
Poodle	7.8	6.0	4.4
Retriever	5.8	4.1	7.0
Total:	100.0 (n = 398)	100.0 (n = 5.164)	100.0 (n = 38.212)

* Pedigree data supplied by the Hungarian Association of Dog Breeders (1989)

Table 4

Assignment of dogs (n = 39) to individual stages of the cycle on the basis of blood plasma E_2 and P concentrations ($\bar{x} \pm SD$)*

Stage of cycle	Number (and %) of animals	Hormone concentration of the blood plasma	
		E_2 (pg/ml)	P (ng/ml)
Anoestrus	15 (38.5)	low 7.4 ± 6.2	low 4.7 ± 2.9
Prooestrus	6 (15.4)	high (rising) 60.3 ± 51.3	low 2.7 ± 1.3
Oestrus	8 (20.5)	high (declining) 42.2 ± 20.5	high (rising) 21.0 ± 8.6
Metoestrus	10 (25.6)	low 7.7 ± 4.9	high (declining) 20.2 ± 6.0

* criteria of classification: E_2 higher or lower than 18 pg/ml, P higher or lower than 10 ng/ml

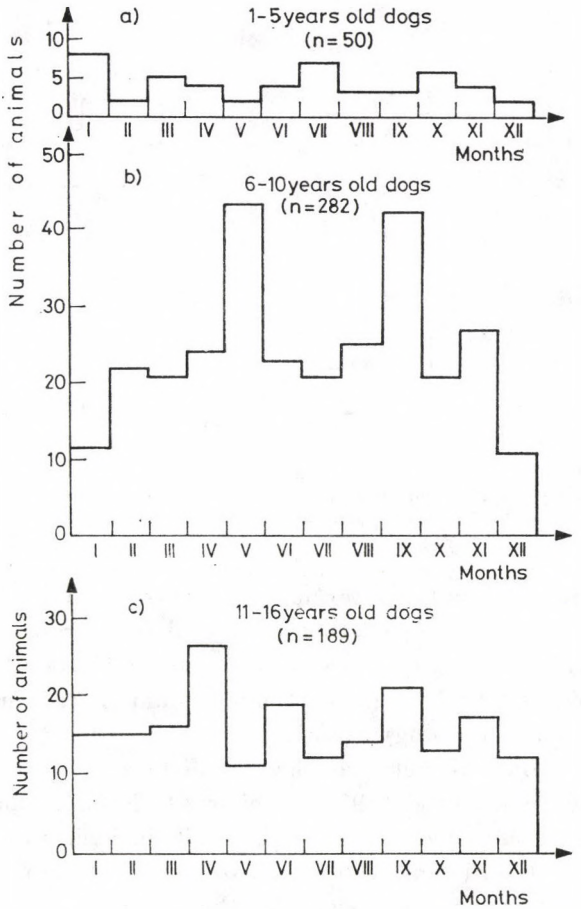

Fig. 5. Seasonal distribution of canine mammary tumours (n = 521) in different age groups in the years 1985 through 1989. *a)* 1–5 years old dogs (n = 50); *b)* 6–10 years old dogs (n = = 282); *c)* 11–16 years old dogs (n = 189)

attention as they supply baseline data on the one hand and call attention to certain correlations on the other.

The data available on dogs presented at the clinics of the University of Veterinary Science of Budapest in 1985 give information, with certain limitations, on the prevalence of canine mammary tumour in Hungary (Table 1). At that time 0.7% of all canine patients had mammary tumour. As animals suspected to have mammary tumour are usually sent to the Clinic of Obstetrics, there 9.5% of all canine patients were found to have mammary tumour.

According to B. Kovács and Somogyvári (1968), 9.1% of the more than 30 thousand dogs treated at Clinic of Surgery of the University of Veterinary Science between 1914 and 1964 had tumours. Incidences as high as 16.3%

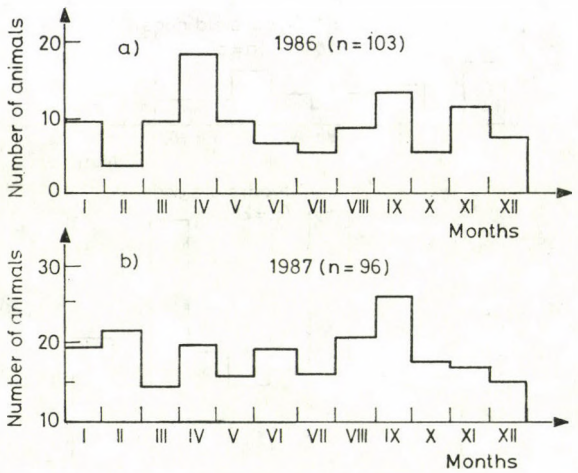

Fig. 6. Monthly distribution of canine mammary tumours in 1986 and 1987 (average age of patients: 10 years)

have also been reported in the veterinary literature (Lechner, 1958). B. Kovács and Somogyvári (1968) reported that within all tumourous dogs the ratio of females was 38.7%. Mammary cancer constituted 13.9% of all canine tumours. Überreiter (1960) and Lechner (1958) found mammary tumours in 35.3% and 40%, respectively, of the dogs examined.

It is an established fact that the risk of tumour formation usually increases with age (Saluja et al., 1974). Similarly to humans, mammary tumour usually develops at an old age in the dog, too: its incidence rate increases after the 6th year of life (Dorn et al., 1968). According to Dietzmann (1960), the peak of mammary tumour incidence occurs at the age of 10 years. Other authors suggest a somewhat younger age for that (Lechner, 1958). The maximum incidence of *all* canine tumours is demonstrable around the age of 8 years (B. Kovács and Somogyvári, 1968). Our statistics made at the Clinic of Obstetrics in the years 1985 through 1989 indicate that the highest incidence of canine mammary tumour occurs in 10-year-old dogs, although the highest relative frequency with respect to female canine patients of the same age was found in the 15th year of life.

The age distribution of all tumour cases as given by B. Kovács and Somogyvári (1968) differs from that found by Szokolóczy (1974) and by us for canine mammary tumours (Fig. 2). The mode of the curve depicting overall tumour incidence in domestic animals is shifted forwards by 2–2.5 years and a high number of tumour cases occur in animals younger than 3 years. This difference can be attributed to benign tumours of viral origin, primarily papillomas (skin lesions) or, possibly, leucosis complexes which frequently occur in young animals (Tamás, 1986).

In humans, the size of mammary tumour is known to correlate with the sexual cycle (Eckhardt, 1977). The seasonal peaks consistently found in the number of canine mammary tumours during the spring and the autumn cycle in the five-year period of study may be accounted for by the same phenomenon. Although the time the animal is presented at the clinic does not necessarily coincide with the incipient stage of the disease, the above data indicate that in the "peak periods" the tumours grew and attracted the owners' attention.

In 10- to 13-year-old dogs the number of tumour cases showed a pronounced rise at the time of the two oestruses occurring during a year. Based upon the hormone levels measured, old dogs which appeared sexually inactive could not be considered to be in a state of sexual rest, although they corresponded to 56–68 years old women as shown by comparative studies (Lebeau, 1953). This is consistent with the findings of Angeli et al. (1989) who demonstrated that oestrogen receptor (ER) concentration showed a circannual rhythm, with a spring acrophase, in a post-menopausal human patient between 1982 and 1988. No circannual rhythm was demonstrable in the progesterone receptor (PR) concentration.

The blood plasma hormone levels measured by us are in good agreement with those published in the literature (Concannon, 1986) for E_2 (5–100 pg/ml) and P (1–85 ng/ml). As blood sexual steroid levels depend on the stage of cycle, P varies between rather wide ranges (Withrow and Susaneck, 1986). It is to be hoped that in the future the knowledge of the hormonal status of animals during different stages of the cycle will allow more successful comparative studies on tumour formation, development and therapy in animals and humans. Further studies are needed in this field, as the dioestrous nature of dogs restricts their use as model animals in the research of mammary tumour therapy in humans.

The differences found in the percentile proportion of individual breeds within all canine patients and within those with tumour call attention to possible breed dispositions. Purebred animals are known to show a higher incidence of tumours (Dorn et al., 1968). E. g. in boxers and terriers mesenchymal tumours, mainly mastocytomas, were reported to be more frequent (Tamás, 1986). The existence of such differences is indicated by the fact that the fox terriers studied by Mialot (1982) in low numbers were oestrogen and progesterone receptor negative whereas the boxers receptor positive. Our observations support the data available in the literature (Heinen, 1976) and complement them with new findings, e.g. the high susceptibility of pulis to mammary tumour. The introduction of regular screening tests of the Hungarian pulis for mammary tumour is worth considering.

The results achieved in the demonstration of human sexual steroid receptors have been insufficiently utilized in the veterinary practice (Sandersleben, 1968), though dogs show a receptor positivity which roughly equals that

of humans (Boldizsár et al., 1991). This insufficiency is due to the fact that receptor determination is not easily accessible yet and the effect of antioestrogenic drugs has not been studied in dogs. Therefore, in harmony with the Hungarian and international practice, the surgical removal of canine mammary tumours is absolutely justified. Any delay with the surgical intervention may result in metastasis or makes the tumour inoperable (Withrow and Susaneck, 1986). The above-mentioned results of the different therapeutic approaches justify the effort to perform, when needed (e.g. in the case of valuable breeding animals), hormone receptor determination, histological examination and the best conservative treatment selected on the basis of the former.

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Acta Veterinaria Hungarica 40 (1—2), pp. 89—97 (1992)

STUDIES ON CANINE MAMMARY TUMOURS II. OESTRADIOL AND PROGESTERONE RECEPTOR BINDING CAPACITY AND HISTOLOGICAL TYPE

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Tissue samples taken from the mammary gland of 42 dogs (age: 6 to 12 years) were examined. Thirty-eight samples showed neoplasia: 36 were epithelial while the remaining 2 proved to be connective tissue tumours. Thirty-four % of the neoplasms were new benign tumours (most frequently adenoma and fibroadenoma) and 66% were malignant ones (mainly adenocarcinoma). The oestrogen receptor (ER) and progesterone receptor (PR) binding capacity was determined on 21 tissue samples using the method of EORTC (1980). The connective tissue tumours and non-tumourous tissues contained no sexual steroid receptor. 71.4% of all tissue samples contained receptors. 61.9% of the samples was ER^+, 42.8% was PR^+, 33.3% contained both receptors, 28.6% was only ER^+ and 9.5% only PR^+. The average ER and PR binding capacity was 120.3 (5.0–622.8) and 266.7 (92.3–475.0) fmol/mg cytosol protein, respectively. No difference in receptor positivity was demonstrable between the benign and the malignant tumours. PR negativity accompanied by ER positivity was more common in the case of benign tumours. ER binding capacity tended to be correlated with age: this correlation could be described with a hyperboloid regression curve ($r = -0.5931$; $0.06 > p > 0.05$).

Key words: Dog, mammary tumour, blood plasma 17β-oestradiol, progesterone, steroid receptors

Mammary tumour is the commonest neoplastic disease of dogs. According to Moulton et al. (1970) and Schlatterer and Wiegner (1980), tumour growth is oestrogen dependent already at a cytosol protein receptor capacity of 3 fmol/mg or more.

The study of oestrogen receptors (ER) occurring in canine mammary tumours was started in the mid-seventies. This was later followed by investigations into the progesterone receptors (PR). More recently, studies on androgen receptors have been initiated (D'Arville and Pierrepoint, 1979; Elling and Staimer, 1981; Elling and Ungemach, 1981, 1983; Evans and Pierrepoint, 1975; Hamilton et al., 1977; McEwen et al., 1982; Mialot et al., 1982*a*, *b*; Monson et al., 1977; Schlatterer and Wiegner, 1980).

Recent data of the literature suggest that ER and PR binding capacity depends on the plasma steroid level and, indirectly, also on the cycle (Caselitz, 1984; Gelly and Pasqualini, 1986; Számel et al., 1983). The aim of this work was to determine the ER and PR binding capacity of the tumour tissue and the histological type of the tumours. Furthermore, we looked for correlations, if any, between tumour incidence and the above parameters.

Materials and methods

For histological examination the samples were fixed in formalin, embedded in paraffin and stained with haematoxylin and eosin. The tissues were classified according to Bulletin 50 of the WHO (Hampe and Misdorp, 1974). The dogs used in the study (n = 42) belonged to different breeds and their age was between 6 and 12 years (average age: 8.5 years).

Receptor binding capacity was determined by the standardized method of the EORTC (1980), using tissue samples (n = 21) pulverized at −196 °C temperature. The samples were homogenized in TRIS-EDTA buffer containing 10% glycerol (pH 7.4) and the cytosol fraction was separated by ultracentrifugation (100,000 *g*, 2 °C, 1 h). The cytosol was incubated with ^{3}H-17ß-oestradiol or ^{3}H-ORG-2058 (a synthetic gestagen) at 2 °C for 18 h. A 1000-fold excess of diethylstilboestrol or nonlabelled ORG-2058 was used as inhibitor. The free and the receptor-bound labelled ligands were separated by a dextran-coated carbon (DCC) technique. ^{3}H activity was measured with a liquid scintillation instrument. The number of specific receptor binding sites was evaluated by Scatchard's analysis (1949). Five and 10 fmol/mg cytosol protein was taken as the limit value of ER and PR binding capacity, respectively. The protein content of the cytosol was determined according to Lowry et al. (1951).

The means, standard deviations and extreme values of the results were given. The means were compared by Student's two-tailed *t* test. The correlation of receptor binding capacity with age was studied by calculating hyperbolic regression.

Results

Thirty-eight out of the 42 tissue samples subjected to histopathological examination proved to be tumourous: 34.2% were benign and 65.8% were malignant tumours. The commonest malignant tumour was adenocarcinoma, while of the benign tumours adenoma and fibroadenoma occurred most frequently (Table 1).

Tumours of epithelial origin occurred in the largest number (in 36 cases). Two samples turned out to be connective tissue tumours, while the remaining 4 did not prove to be neoplastic.

In the second part of the work the oestrogen and progesterone receptor status of the tumour tissues was examined. The connective tissue tumours and non-tumourous tissues did not contain steroid hormone receptors. 28.6% of the samples contained only ER, 9.5% only PR, while 33.3% contained both receptors. Consequently, 61.9% of the samples was ER$^+$, 42.8% was PR$^+$, and 71.4% contained either ER or PR. The average ER and PR binding capacity was 120.7 (5.0–622.8) and 266.7 (92.3–475.0) fmol/mg cytosol protein, respec-

tively, which are values typical of tumour tissues (Table 2). (The K_d values indicative of receptor binding were in the magnitude of 10^{-10} M). No difference in receptor positivity was demonstrable between the benign and the malignant tumours. As regards the effect of age, both ER and PR positivity and negativity occurred among 7–11 years old animals.

Table 1

Histological classification of mammary tumours

	Histological type	No. of animals
Malignant	Solid carcinoma, simple type	3
	Solid carcinoma, complex type	3
	Tubular adenocarcinoma, simple type, differentiated	8
	Tubular adenocarcinoma, simple type, not differentiated	1
	Tubular adenocarcinoma, complex type	1
	Papillary cystic adenocarcinoma, simple type	7
	Mucinous carcinoma	1
	Fibrosarcoma	1
Benign	Adenoma, simple type	1
	Adenoma, complex type	4
	Duct papilloma	1
	Pericanalicular fibroadenoma	5
	Benign fibroadenoma, mixed tumour	1
	Fibroma	1
Non-tumourous	Noninflammatory lobular hyperplasia	1
	Scar tissue	1
	Lymph node	1
	Necrotic tissue	1
Total:		42

Table 2

ER and PR binding capacity of the tumour tissue (n = 21; protein content of the cytosol fraction: 2–8.5 mg/ml)

Receptor		Receptor-positive tissue (%)
ER+ PR+		33.3
ER+ PR−		28.6
ER− PR+		9.5
ER− PR−		28.6
ER+, total	61.9	$\bar{x}$ = 120.3 (5.0–622.8) fmol/mg cytosol protein
PR+, total	42.8	$\bar{x}$ = 266.7 (92.3–475.0) fmol/mg cytosol protein

No difference was demonstrable between benign and malignant tumours in ER binding capacity. PR negativity accompanied by ER positivity was more common in the case of benign tumours.

Correlations, if any, between receptor content and age were also investigated. While no such correlation was demonstrable for PR, ER binding capacity tended to be correlated with age, showing a hyperboloid regression curve ($r = -0.5931$; $0.06 > p > 0.05$; Fig. 1).

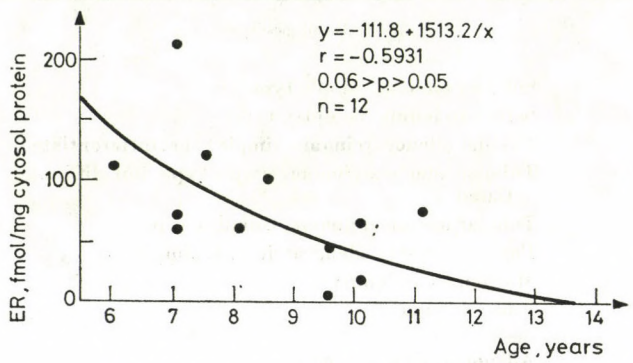

Fig. 1. Correlation of ER content with age

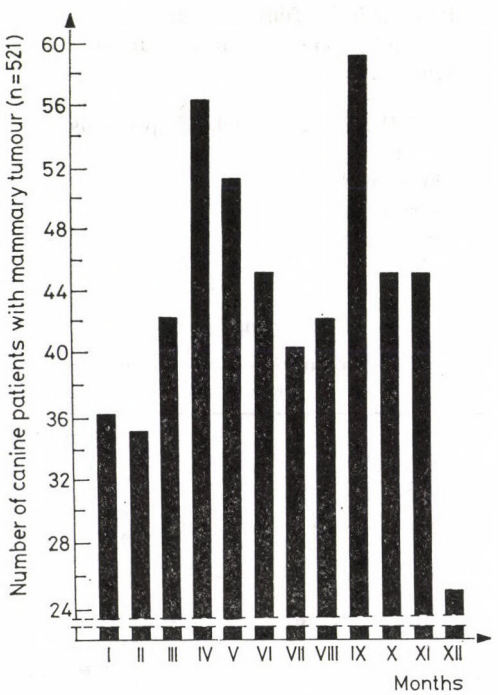

Fig. 2. Incidence of canine mammary tumours (n = 521) in different months of the year

Dogs presented at the Clinic of Obstetrics, University of Veterinary Science, with the suspicion of mammary tumour in the years 1986 through 1989 (n = 521) showed a characteristic seasonal distribution. Two peaks were observed: one in the spring, in April (May) and another in the autumn, in September (Fig. 2).

Studying the patient material by age, the two seasonal peaks in mammary tumour incidence are consistently demonstrable in dogs more than 6 years old. These two peaks were detectable also in 11- to 16-year-old, sexually inactive, apparently post-menopausal bitches, especially in those aged 14–15 years.

No significant differences could be observed in average mammary tumour incidence by year. The number of patients increased neither in the year of the Chernobyl catastrophe (1986) nor thereafter. Statistical data on the incidence of human breast cancer indicate no direct correlation either (Central Statistical Office, 1985–1989).

Discussion

Different authors hold divergent views on the distribution of canine mammary tumours by malignancy. In this study, two-thirds of the tumours examined were malignant. Malignant tumours occurred most frequently in 11-year-old bitches. Of the 360 histopathologically examined tumours described by B. Kovács and Somogyvári (1968) from both sexes of dogs, 67.2% were malignant and 32.8% benign. In the studies of Caselitz (1984) as well as Knoll and Unger (1983), half of the tumours examined proved to be malignant. According to Misdorp (1967), Cotchin (1967), Sandersleben (1968) and Owen (1986), two-thirds of canine mammary tumours are benign and metastases are less common than in human breast cancer. In the opinion of Dobberstein and Matthias (1959), only 10% of the cases are really malignant.

In our study, adenocarcinoma proved to be the commonest malignant tumour, whereas the most frequently occurring type of benign tumour was adenoma. This is consistent with the findings of most authors (Withrow and Susaneck, 1986).

The histopathological type of tumours is studied first of all to prognosticate survival and the success of surgical and other therapeutic interventions on the one hand and because of its postulated correlation with the receptor status on the other.

Endocrine therapy has a fair chance of success in the presence of sexual steroid receptors. In the case of simultaneous ER and PR positivity hormonal treatment usually results in remission. Although receptors may occur in normal mammary tissue, their quantity cannot be measured (Caselitz, 1984; Elling and Ungemach, 1983; Martin et al., 1978). ER was reported to occur more frequently in benign tumours (Monson et al., 1977). Certain investigators

(Elling and Ungemach, 1981, 1983; Mialot et al., 1982*a*, *b)* failed to find any difference in receptor positivity between benign and malignant tumours, while others (Caselitz, 1984) reported that receptor content decreased parallel with the increase of malignancy. In this study, no difference was found between benign and malignant tumours in receptor positivity. The connective tissue tumours and scar-tissues contained no steroid hormone receptor. This is consistent with data of the literature that tumour tissues of mesenchymal origin contain low amounts of sexual steroid receptor and are less susceptible to hormonal influences than are those of epithelial origin. In mammary adenomas and fibroadenomas the receptors can be found predominantly in the epithelium (Caselitz, 1984). Ninety-two per cent of the canine mammary tumours examined by McEwen (1982) were epithelial tumours which showed receptor positivity while the non-epithelial tumours proved to be receptor negative. Mixed tumours were characterized by lower receptor-binding capacity than adenocarcinomas.

Of canine mammary tumours examined by Mialot et al. (1982*a)*, 48.2% were ER^+PR^+, 0.8% ER^+PR^- and 7.3% ER^-PR^+. Thus, 63.5% of the dogs were found to have receptors. This is somewhat lower than the frequency of ER or PR occurrence (primarily ER^+PR^-) obtained by us (71.4%) in the tumour tissues examined. PR positivity can be found especially in well-differentiated tumours of low malignancy (Tulusan et al., 1982). PR negativity with ER positivity is common in postmenopausal women suffering from breast cancer (Caselitz, 1984). The incidence of PR positivity in canine mammary tumour has been reported to be 23–62% (Elling and Ungemach, 1983). PR positivity without ER positivity is more common in dogs (20%) than in humans.

The values of receptor binding capacity found by us (Table 2) are higher than those reported in the literature [ER: 62.7 (13–129) and PR: 30.6 (5–80.9) fmol/mg cytosol protein] by D'Arville and Pierrepoint (1979); however, the large distance between the extreme values suggests that extremely high variation must be reckoned with.

At the beginning of the 19th century, Cooper found that tumour size was related to the cycle (Caselitz, 1984). The same correlation may account for the observation that in the five-year period of study the number of tumourous dogs was consistently higher during the spring or autumn cycle than in other months of the year. Presentation of the affected animal at the clinic suggests that the tumour may have considerably increased in size in those periods.

In benign mammary tumours of women, ER content is in direct proportion to the degree of hyperplasia (Jacquemier et al., 1982). Others (McEwen et al., 1982; Rosen et al., 1975) could not find any relationship between ER content and tumour size. According to Mialot et al. (1982*a)*, canine tumours exceeding 10 cm in size are characterized by a lower incidence (%) of ER and PR positivity than those measuring 5 cm or less. Although Mialot did not find

these results to be conclusive, they may well explain Csenki and Tory's (1983) observation that tamoxifen treatment was successful against small tumours of old dogs, as the success of treatment with the antioestrogen tamoxifen is known to be related to receptor positivity.

It is an accepted fact that the risk of tumour formation increases with age (Saluja et al., 1974). Therefore, we supposed, but could not prove, that receptor positivity decreases with age.

Although receptor positivity, rather than receptor binding capacity, is of decisive importance with a view to the prognosis, oestrogen receptor content and age tended to be correlated. From the age of 6 years the degree of this correlation undergoes a hyperbolic decrease. On the basis of this correlation, theoretically the tissue would cease to contain ER at the age of 13.5 years, when the number of mammary tumours shows a striking rise (as expressed in % of dogs of the same age, examined for other diseases). PR positivity is not age dependent (Caselitz, 1984). No correlation between PR content and age was demonstrable in this study either. According to the histochemical studies of Caselitz (1984) and studies performed by others (Kardos and Guidotti, 1988), blood progesterone and oestrone concentrations markedly influence the receptor content. In part one of this paper (Boldizsár et al., 1992; see page 75 in this issue) we reported the blood plasma progesterone and 17 β-oestradiol concentrations of the dogs examined. Based upon data of the literature cited above, we assumed that plasma steroid level was in a negative correlation with receptor binding capacity; however, the data are insufficient to enable us to take up a definite position on this question.

Several decades ago, the unsolved questions of tumour research emphasized the importance of comparative oncological studies the organization of which has been urged on by the WHO since the 1960s (Cotchin, 1967). When evaluating experience and data obtained in animals, one has to start from the principle that the tumours of animals do not differ from those of humans in their morphological, histological and biological properties (metastasis, recurrence) and effects on the whole organism. However, the existing differences, and the fact that the behaviour of tumours of similar tissue composition may differ by species, must not be disregarded. According to Elling and Staimer (1981), canine mammary tumours have unique properties in that they consist of proliferations of epithelial and myoepithelial components. Receptor profile cannot be linked with a given histological diagnosis. Even morphologically uniform tumours lack a uniform receptor profile. A receptor-positive tumour tissue may contain receptor-negative cells, and conversely. In contrast to those of humans, the mammary tumour cells of dogs contain PR also in their nuclei. While in humans the myoepithelium contains little or no ER, in dogs ER- and PR--dependent fluorescence is at least as strong in the myoepithelium as in the glandular epithelium (Elling and Staimer, 1981).

The facts mentioned above regarding the success of different therapeutic approaches justify the effort to perform, when needed (e.g. in the case of valuable breeding animals), receptor and histological type determination and the best medicinal, conservative treatment selected on the basis of the former.

It is known that after the Chernobyl nuclear reactor catastrophe primarily radioiodine, cesium and strontium contamination had to be reckoned with. Secretion of calcium and iodine with the milk exposed the mammary gland to a high risk of disease (Kirchhoff, 1987). The FAO urged all along that after nuclear catastrophes the food chains should be monitored (FAO, 1987). The food chain of man (Kirchhoff, 1987) only partly agrees with that of the dog. Thus, while the quickly developing radioisotope contamination of plants did not pose a direct threat to carnivores through their food intake, contamination via the drinking water was a distinct possibility. Our data do not indicate a rise in the incidence of canine mammary tumours following the Chernobyl catastrophe.

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SULFONAMIDE RESIDUES IN EGGS

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In two series of experiments lasting 3 days each, laying hens were medicated with sulfonamides via the drinking water. In the first experiment 8 laying hens were given sulfaquinoxaline (SQ) at a dose rate of 400 mg/l, while in the second trial 16 laying hens received a 3 : 5 : 5 sulfonamide mixture containing sulfaquinoxaline (SQ), sulfadimidine (SDI) and sulfamerazine (SMN), at a dose of 390 mg/l. According to the water consumption data, the hens' daily sulfonamide intake was 53.6 and 56.9 mg/kg body mass, respectively. Eggs laid during and in the first 10 days after the treatment were collected and assayed for sulfonamide residues by spectrophotometry. The detection limit of the method was 0.16 mg/l and the recovery percentage was between 70 and 80%.

Sulfonamide was found to appear already in eggs laid after the first day of treatment. The absorption half-life of the drug was 0.4–0.6 day in the egg-white and 0.93–1.08 day in the egg-yolk. Peak drug level in the egg-white was measured on the last day of medication, while in the egg-yolk within 3 days after the end of treatment. The residue measured in the yolk was 13–16% of that found in the egg-white. Acetylated sulfonamide could be measured in the yolk for 3 days after the treatment: its level reached 15%.

On the basis of the elimination rate, complete elimination of sulfonamides requires at least 5.2–7.4 days. Therefore, observance of the generally accepted withdrawal time of 10 days is indispensable.

Key words: Sulfonamide, residue, laying hen, egg, elimination, pharmacokinetics

One of the most important conditions required for the marketing and use of veterinary pharmaceuticals is their elimination-kinetic and residue study. Drugs administered to laying hens inevitably appear in the yolk and egg-white. The residue level depends on the properties of the active ingredient, the dose applied and the route of application.

Sulfonamides have been used in poultry species for more than four decades. They are administered to laying hens and, occasionally, to broiler chickens as anticoccidial and antibacterial agents.

The pharmacokinetic properties of different sulfonamides vary widely by the type of preparation and the animal species treated (Dorrestein, 1988). According to data of the literature, the elimination half-life (t_{50e}) of sulfadimidine in laying hens is 16.8 $\pm$ 7.8 h (Losch, 1980). Values reported for the elimination half-life of sulfadimidine orally administered to Leghorn chickens varied from 18.3 h (Blom, 1975) through 15.3 h (Reddy, 1988) to 1.4 h (Löscher et al., 1990). In turkeys the t_{50e} was reported to be 7.1 h (Heath et al., 1975). The t_{50e}

of sulfanilamide in chickens is 13.3 ± 2.5 h (Blom, 1975) while that of sulfaquinoxaline is 22.2 ± 1.9 h in chickens (Blom, 1975) and 19.24 ± 2.3 h (Romváry and Horvay, 1976) or 30 h (Epstein, 1989) in geese.

The distribution of sulfonamides in eggs has been studied by several researchers who have established that the elimination of sulfonamides via eggs takes at least 10 days as from the end of medication (Blom, 1974, 1975; Nouws, 1976; Nagata et al., 1989). Traces of acetylated metabolites can be detected both in the egg-white and in the yolk for 3 days after the medication. Diurnal variations in the elimination rate have also been reported (Kietzmann, 1982).

In this work, the elimination dynamics via the eggs of sulfaquinoxaline and a 3 : 5 : 5 combination of sulfaquinoxaline, sulfadimidine and sulfamerazine administered to laying hens in the drinking water was studied. The aim of the experiments was to determine the residue levels of sulfonamides in the egg-white and yolk after medication via the drinking water.

Materials and methods

Two series of experiments were carried out. In the first trial, 8 Tetra SL laying hens were medicated with sulfaquinoxaline (SQ) via the drinking water provided *ad libitum*, at a dose of 400 mg/l for 3 days. The eggs laid during the treatment and in the first 10 days after the end of medication (a total of 73 eggs) were collected. By indirect measurement of the drinking water consumption the average daily drug intake of the birds (average body mass: 2.1 ± 0.1 kg) was found to be 49.4–57.9 mg/kg body mass.

In the second experiment, 16 laying hens (average body mass: 2.0 ± 0.03 kg) were treated with a 3 : 5 : 5 sulfonamide combination containing sulfaquinoxaline, sulfadimidine and sulfamerazine at a dose of 390 mg/l for 3 days. On the basis of the drinking water consumption the daily drug intake was found to be 56.9 mg/kg body mass. Thirty-nine and 81 eggs were collected during and after the treatment, respectively.

The design of the experiments is shown in Table 1.

Free and acetylated sulfonamide concentrations were measured spectrophotometrically by the method of Blom (1975). By this procedure sulfonamide residue levels of 0.167 and 0.161 mg/l were detected in the egg-white and egg-yolk, respectively. The recovery rate of the procedure was 74.3 ± 6.0% for the egg-white and 81.4 ± 5.2% for the yolk, respectively.

The results were analyzed with the help of a one-compartment pharmacokinetic model, by the method of Ritschell (1973), using an Aztech 286 computer and a MEDUSA programme.

Table 1

Design of the experiments

Group	Drug	Concentration in the drinking water (mg/l)	n	Animals	Before medication (3 days)		During medication (3 days)			After medication (10 days)	
				kg b.m. $x \pm s_x$	dr. water ml/kg/d	eggs n	dr. w. ml/kg/d	drug mg/kg/d	eggs n	dr. water ml/kg/d	eggs n
1	SQ	400	8	2.1 ± 0.1	—	—	134 ± 7	53.6	17	—	56
2	SQ SDI SMN	390	16	2.0 ± 0.03	122 ± 2	39	146 ± 6	56.9	39	121 ± 5	81

SQ = sulfaquinoxaline; SDI = sulfadimidine; SMN = sulfamerazine

Table 2

Changes in sulfonamide residue levels of eggs laid by hens treated with sulfaquinoxaline-Na administered in the drinking water, during and after the medication

Eggs		During medication			After medication							
		Day 1	Day 2	Day 3	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8
Egg-white	x	2.14	3.28	6.65	8.53	7.08	3.05	0.91	0.29	0.19	0.17	<0.17
	$\pm s_x$	0.11	0.23	0.17	0.53	0.34	0.08	0.21	0.05	0.01	0.01	
	max.	2.29	3.68	6.84	11.14	7.71	3.12	1.40	0.33	0.27	0.19	<0.17
	min.	2.04	2.90	6.12	6.72	6.49	2.98	0.56	0.27	0.17	0.17	<0.17
Egg-yolk	x	0.19	0.51	0.77	1.10	1.12	1.20	1.27	0.76	0.55	0.36	0.17
	$\pm s_x$	0.01	0.07	0.08	0.16	0.11	0.13	0.17	0.09	0.11	0.01	0.11
	max.	0.28	0.54	0.83	1.58	1.30	1.31	1.81	0.82	0.63	0.45	0.23
	min.	0.17	0.48	0.72	0.99	1.06	1.01	0.83	0.68	0.40	0.36	<0.17

Results

Administration of sulfaquinoxaline in the drinking water resulted in a continuous rise of drug concentration in the egg-white. Drug residue levels reached a level of 6.1–6.8 mg/l already after the first two days of medication. Drug concentration in the egg-white started to decrease on day 2 after the end of medication; however, a marked reduction occurred only on days 4–5 when the sulfonamide level reached 0.3–0.9 mg/l. By the method applied by us, no sulfonamide could be detected in the egg-white on day 8 after the treatment.

Elimination of SQ via the egg yolk could be observed already during the treatment; the residue level was, however, only 0.1–0.8 mg/l. In the yolk of eggs laid after the end of medication, the residue levels rose steadily up to day 4 but did not exceed 1.8 mg/l. From day 5, the SQ content of the yolk began to decrease and became undetectable by our method by day 9–10. Changes of the residue levels are shown in Table 2 and Fig. 1.

The changes in drug residue levels showed a similar tendency also when the sulfonamide combination was used. In the egg–white, sulfonamide reached a concentration of 6.0 ± 0.6 mg/l already after the first day of medication. Individual variation was, however, considerable: the highest level was 10.1 mg/l and only one out of the 15 eggs examined was residue free. On the subsequent days of treatment, sulfonamide in the egg-white reached a concentration as high as 23.5 mg/l, though its average level was 10–11 mg/l.

After the end of medication, drug residue level in the egg-white significantly decreases: it drops to 3.7 ± 0.3 mg/l by the 2nd day. No sulfonamide could be detected by our method in the egg-white of eggs laid on day 3 after the medication.

Sulfonamide appeared also in the egg-yolk after the first day of medication (average level: 1.2 ± 0.29 mg/l), though in 3 eggs no drug residues were detectable. On the subsequent days of medication the sulfonamide concentration of the egg-yolk continuously increased and reached a maximum of 5.7 mg/l. After the termination of treatment, residue level of the yolk decreased and by day 5 it became undetectable by the method applied.

Acetylated sulfonamide derivatives could be measured only in the yolk on the last day of treatment (14.8%) and between days 1–3 after the end of medication when their level ranged between 13.8 and 15.5%.

The residue levels of the sulfonamide combination are shown in Table 3 and Fig. 2.

The results were analyzed using a one-compartment kinetic model. The kinetic parameters are presented in Table 4. It can be seen that different sulfonamides applied for a similar period of time at a nearly identical dose became absorbed into the egg-white and the yolk at a similar rate (t_{50e} for the egg-white 0.69 and 0.4 day while for the yolk 0.93 and 1.08 day, respectively). In the case

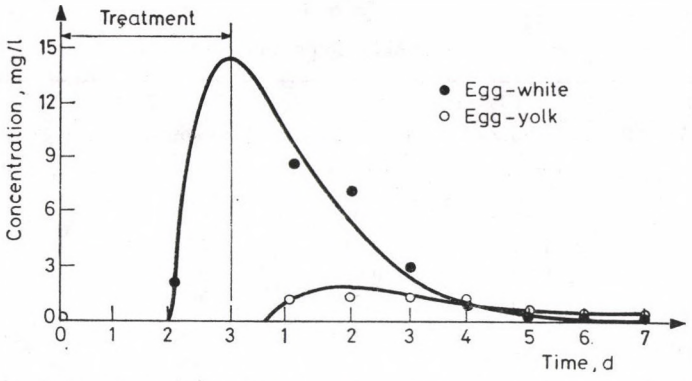

Fig. 1. Sulfaquinoxaline residues in eggs (one-compartment per os model)

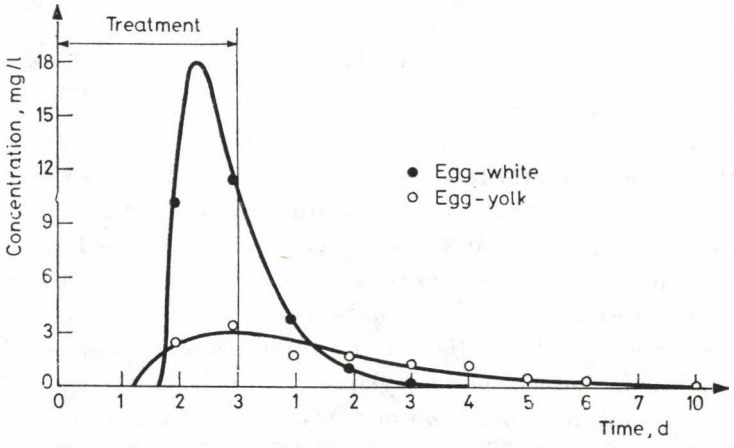

Fig. 2. Sulfonamide residues in eggs of laying hens treated with a sulfonamide combination (one-compartment per os model)

Table 3

Changes in sulfonamide residue levels of eggs laid by hens treated with a sulfonamide combination administered in the drinking water, during and after the medication

Eggs		During medication			After medication					
		Day 1	Day 2	Day 3	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
Egg-white	x	6.09	10.11	11.35	3.70	1.18	<0.17	<0.17	<0.17	<0.17
	$\pm s_x$	0.6	1.4	1.0	0.3	0.18				
	max.	10.1	23.5	16.9	5.22	1.89				
	min.	<0.17	5.5	7.2	2.52	0.18				
Egg-yolk	x	1.22	2.26	3.21	1.74	1.45	1.15	1.09	0.42	<0.16
	$\pm s_x$	0.3	0.4	0.4	0.5	0.3	0.2	0.1	0.12	
	max.	3.03	5.70	5.68	3.85	2.50	1.83	1.88	0.63	
	min.	<0.16	0.75	0.98	0.23	0.16	0.51	0.31	<0.16	

Table 4

Pharmacokinetic parameters

Parameter	Sulfaquinoxaline (400 mg/l; 53.6 mg/kg body mass; 3 days)		SQ + SDI + SMN (390 mg/l; 56.9 mg/kg body mass; 3 days)	
	egg-white	egg-yolk	egg-white	egg-yolk
C_0, mg/kg	40.02	5.08	56.86	8.10
K_a d^{-1}	1.02	0.74	1.57	0.64
t_{50a} d	0.69	0.93	0.44	1.08
K_e d^{-1}	1.05	0.765	2.12	0.65
t_{50e} d	0.66	0.91	0.33	1.07
t lag d	1.95	3.63	1.85	1.33
C_{max}, mg/kg	14.45	1.84	17.88	2.97
t_{max} d	2.91	4.96	2.40	2.88
AUC mg/l/d	30.21	7.08	29.56	12.18
MRT d	4.16	5.78	2.68	4.05
r	0.9375	0.9284	0.9359	0.9284

of SQ administration in the drinking water the peak residue level developed later: in the egg-white on the last day of treatment, while in the yolk 2 days after the medication. When using the sulfonamide combination, the peak residue level in both the egg-white and the yolk occurred around the last day of medication. The elimination of SQ from the egg-white was delayed (t_{50e} = 0.7 day), while that of the sulfonamide combination took only 0.3 day.

The tolerance level specified by the Bundesgesundheitsamt (BGA, 1984) for sulfonamides including their acetylated metabolites is 0.1 mg/kg. According to our results, after administration of SQ in the drinking water this tolerance level in the egg-white and yolk is reached in 4.7 and 7.4 days, respectively. With the sulfonamide combination, the above tolerance level is reached in 2.5 and 5.2 days in the egg-white and yolk, respectively.

Discussion

Sulfonamides become absorbed well after both oral and parenteral administration. As a result of this rapid absorption, peak blood concentrations develop within 3–5 h (Lüders et al., 1974).

The distribution of sulfonamides is relatively homogeneous: the residue levels measured in the tissues amount to 20–40% of the blood levels; however, residues measurable in the liver and kidney consistently exceed the blood plasma levels (Atef et al., 1978).

Of the sulfonamide metabolites, the amount of acetylated derivatives varies by animal species and preparation. In laying hens the acetylated derivative of sulfadimidine amounts to 3.5–6.9% while that of sulfaquinoxaline only

to 0.6% (Blom, 1975). The ratio of metabolites is low also in pigeons (Dorrestein, 1988). Acetylated metabolites are transmitted into the egg-white and egg-yolk: those of sulfadimidine amount to 3–3.4% and 0.7–1.2% in the egg-white and egg-yolk, respectively. The quantity of acetylated sulfaquinoxaline is much lower: 1.8% in the egg-white and 0.5% in the yolk.

In sexually mature laying hens, the maturation of ova takes place in the left ovary in three phases. The first and second phases, lasting about 80 and 60 days, respectively, are followed by a 10-day period of rapid development which results in the formation of the egg-yolk (Siegmann, 1980). In the maturation phase, the increase of follicle size is linear: before ovulation the mass of the follicle is 17 g and its diameter is 37 mm. As a result of the continuous and rapid maturation, the drugs and their metabolities appear, to a different extent, in the eggs, reflecting the actual drug level of the organism. The residue level of the egg-yolk is determined by the actual drug level of the organism 3–5 days before egg-laying, while that of the egg-white depends on the drug concentration of the organism on the day before laying.

Because of the prolonged elimination a rather long withdrawal period is recommended and prescribed. According to Krieg (1966), sulfadimidine became undetectable in the egg-white and the yolk by days 6 and 8 after medication, respectively. Similarly, Stange (1977) proposed a 8- to 10-day withdrawal time for yolk.

Our results show that a daily intake of 54–57 mg/kg body mass sulfonamide with the drinking water produced drug residues both in the egg-white and in the yolk. The maximum level was 14.5–17.9 mg/l and 1.8–3.0 mg/l in the egg-white and the yolk, respectively.

Only 13–16% of the drug residue content of the egg-white appears in the yolk; however, the ratio of acetylated metabolites may reach 13–15%.

The half-life of SQ elimination via the egg-white is 0.66 day (15.84 h), indicating faster elimination than that reported for the blood plasma (22.2 h) by Blom (1975). The t_{50e} value of SQ in the yolk is 0.91 d (21.84 h).

The elimination of the sulfonamide combination (which contained only 24% SQ) was faster in the egg-white (7.9 h) than via the yolk (25.7 h).

The time needed to reach the limit value of 0.1 mg/kg (BGA, 1984) was found to be 5–7 days. Therefore, strict observance of a 10-day withdrawal time after sulfonamide medication is essential.

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COMPARISON OF GENTAMICIN TOXICITY IN NORMAL AND DIABETIC RATS

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Gentamicin treatment caused an elevation in serum urea and creatinine concentrations and ALT activity, associated with pathological changes in the liver and kidney.

The pathological and blood chemistry changes were more severe in diabetic gentamicin-treated than in non-diabetic gentamicin-treated rats.

Alloxan-diabetic rats had lowered blood glutathione concentrations which may have been responsible for the enhancement of gentamicin toxicity in these rats.

Key words: Gentamicin, alloxan diabetes, nephrotoxicity, hepatotoxicity, glutathione, rat

Gentamicin is an aminoglycoside antibiotic effective against a wide variety of bacterial pathogens (Bywater, 1982). The major drawback of gentamicin is its nephrotoxic effect because of which special care must be taken to avoid overdosing. This nephrotoxicity of gentamicin has prompted researchers to evaluate the safety of therapeutic doses of gentamicin in different disease conditions such as liver diseases, kidney dysfunction, endotoxaemia and cystic fibrosis (Frazier et al., 1986; Camps et al., 1988; Jernigan et al., 1988; Frazier et al., 1988; Godson et al., 1988; Zager, 1988).

In this work, the effects of gentamicin in diabetic rats are compared with those exerted in normal rats.

Materials and methods

Drugs. A solution of gentamicin sulphate (Garamycin injection, Schering Corporation, U.S.A.) was prepared in sterile saline at a concentration of 8 mg gentamicin base/ml saline and was given to rats by intraperitoneal injection at a dose of 40 mg/kg body mass/day in two equal doses administered at an interval of 8 h (Houghton et al., 1976). This dose of gentamicin is approximately equivalent to 1 1/3 the maximal therapeutic dose in adult humans (350 mg gentamicin base/70 kg body mass man/day; Harvey, 1980), calculated using the surface area ratio of rat and man as described by Paget and Barnes (1964).

Rats and experimental design. Twenty male albino rats weighing 180 to 200 g were maintained on a commercial rat diet *ad libitum*. Diabetes mellitus was induced in 10 rats by subcutaneous injection of alloxan at a dose of 200

mg/kg body mass. Two weeks later the rats were divided into four equal groups and treated as follows: group 1: diabetic rats given saline; group 2: diabetic rats given gentamicin; group 3: normal rats given saline; group 4: normal rats given gentamicin. Treatment with gentamicin or saline was continued for 10 days.

Samples and analysis. Blood samples were collected 24 h before starting gentamicin or saline treatment and 48 h after the last dose of gentamicin or saline. Each time, two blood samples were collected from each rat. One sample was obtained in a plain test tube and was used for preparation of serum for determination of glucose, urea, creatinine and cholesterol concentrations and alanine aminotransferase (ALT) activities (Diagnostic Kits, BioMerieux Laboratory Reagents and Products, France). The other blood sample was collected in a heparinized test tube kept on ice and used for determination of glutathione (Beutler et al., 1963; Johnson, 1966).

All rats were killed 48 h after the last gentamicin or saline treatment. Tissue specimens were obtained from the liver and kidney, fixed in formalin and histological sections were prepared using paraffin embedding and haematoxylin and eosin staining techniques.

Statistical analysis. The significance of differences between mean concentrations of blood biochemical constituents in the different groups was analyzed using "analysis of variance" and "least significant difference" methods.

Results

Blood glucose concentrations were significantly ($P < 0.01$) higher in alloxan-treated rats (210–290 mg/dl; groups 1 and 2) than in normal rats (63–100 mg/dl; groups 3 and 4).

Blood glutathione concentrations were significantly lower ($P < 0.01$) in diabetic (groups 1 and 2) than in non-diabetic rats (groups 3 and 4). Post-treatment concentrations of glutathione were significantly higher ($P < 0.01$) than pretreatment values in diabetic rats (groups 1 and 2).

Post-treatment serum creatinine and urea concentrations and ALT activities were significantly higher ($P < 0.01$) than pretreatment concentrations in gentamicin-treated rats (groups 2 and 4).

Post-treatment cholesterol concentrations were significantly lower than pretreatment concentrations in both gentamicin-treated and saline-treated diabetic rats (groups 1 and 2). No significant differences were found between post-treatment and pretreatment concentrations of different blood constituents in non-diabetic saline-treated rats (group 3).

Histopathological examination of tissue sections from the liver revealed vacuolization of the cytoplasm of hepatocytes which was mild in degree, af-

fecting only few hepatocytes in saline-treated diabetic rats (group 1) and of moderate degree in both gentamicin-treated groups (groups 2 and 4). Kidney sections showed increased granularity of the cytoplasm, swollen nuclei, detachment of tubular epithelium, and cell debris in the lumina of renal tubules.

No abnormities were observed in non-diabetic saline-treatedrats (group 3).

Discussion

Alloxan-treated rats remained hyperglycaemic throughout the experiment and were left for two weeks before starting administration of different treatments to avoid interference from the toxic effects of alloxan on the liver and kidney (Lopukhin, 1976). Comparison of alloxan-treated and non-treated groups for pretreatment concentrations of creatinine and urea as well as serum ALT activity revealed no statistically significant differences. This finding indicated that liver and kidney functions were normal at the beginning of the experiment in alloxan-treated rats.

Gentamicin administration caused a rise in serum ALT activities in both diabetic and non-diabetic rats. The magnitude of elevation was greater in diabetic than in non-diabetic rats. This was associated with vacuolization of the cytoplasm of hepatocytes in these groups (groups 1, 2 and 4) but not in non-diabetic saline-treated rats (group 3). These findings indicate that gentamicin possesses hepatotoxic effects in normal rats. Diabetes increases the hepatotoxicity of gentamicin.

Gentamicin-induced degeneration and necrosis of the renal tubular epithelium observed in this study are generally similar to those described by Houghton et al. (1976). These pathological changes were more severe and the magnitude of elevation of serum creatinine and urea was greater in gentamicin-treated diabetic rats, findings which indicate that diabetes enhances gentamicin nephrotoxicity.

Previous research has shown that renal dysfunction, cystic fibrosis and presenco of a focus of liver necrosis increase the risk of gentamicin toxicity (Frazier, 1986; Godson et al., 1988; Zager, 1988). Liver cirrhosis and endotoxaemia were suspected but not shown to enhance gentamicin nephrotoxicity in experimental models (Camps et al., 1988; Jernigan et al., 1988).

Enhancement of gentamicin toxicity may be attributed to alterations in pharmacokinetics causing a rise in the blood concentrations of gentamicin in diabetic rats. Another possibility is that the kidney of alloxan-diabetic rats is more sensitive to the gentamicin-induced injury (decreased toxicity threshold). This possibility is supported by the observation that diabetic rats had lowered blood glutathione concentrations (Table 1). Glutathione is known to play a key role in biochemical defence against toxicity-induced tissue injury (Gillette et al., 1974).

Table 1

Concentration of blood constituents in gentamicin-treated and saline-treated rats
($\bar{x} \pm SEM$; $n = 5$)

Blood constituent	Non-diabetic				Diabetic			
	Saline Group 3		Gentamicin Group 4		Saline Group 1		Gentamicin Group 2	
	A	B	A	B	A	B	A	B
Glucose (mg/dl)	75.4 ± 3.1	72.2 ± 3.5	87.4 ± 5.1	83.0 ± 5.4	243 ± 14.3	230 ± 8.6	239 ±13	231 ± 8.5
Glutathione (mg/dl)	37.2 ± 0.6	39.4 ±0.9	33.4 ± 1.1	36.6 ± 1.1	13.0 ± 1.4	28.4** ± 1.6	14.0 ± 0.9	27.0** ± 1.6
Creatinine (mg/dl)	0.83 ± 0.04	0.83 ± 0.04	0.82 ± 0.04	1.1** ± 0.04	0.81 ± 0.04	0.84 ± 0.04	0.83 ± 0.04	1.8** ± 0.04
Urea (mg/dl)	35.4 ± 0.9	35.2 ± 1.2	34.0 ± 1.7	78.2** ± 3.9	33.2 ± 1.8	39.4 ± 2.7	35.4 ± 1.2	131.2** ± 9.1
ALT (U/l)	65.6 ± 4.8	70.0 ± 3.4	71.0 ± 3.9	101.1** ± 3.0	70.4 ± 3.7	75.4 ± 3.7	72.8 ± 5.1	116.2** ± 3.2
Cholesterol (mg/dl)	78.4 ± 4.4	78.2 ± 4.4	77.6 ± 5.4	81.2 ± 8.1	70.6 ± 4.5	53.2** ± 2.1	77.2 ± 3.1	52.2** ± 2.9

A: pretreatment values; B: post-treatment values; ** post-treatment values are highly significantly different from pretreatment values ($P < 0.01$)

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DISPOSITION KINETICS AND DOSAGE REGIMEN OF SULFAPYRIDINE IN BUFFALO *(BUBALUS BUBALIS)*

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The disposition kinetics and dosage regimen of sulfapyridine were studied in buffalo calves following a single intravenous dose of 100 mg/kg. Distribution half-life ($t_{1/2\alpha}$) elimination half-life ($t_{1/2\beta}$) and Vd (area) was 0.181 ± 0.008 h, 13.4 ± 0.52 h and 0.59 ± 0.03 L kg^{-1}, respectively. Total body clearance, which represents the sum of all clearance processes, and tissue/plasma (T/P) ratio were calculated to be 31.1 ± ± 2.28 ml kg^{-1} h^{-1} and 2.25 ± 0.09, respectively. A satisfactory intravenous dosage regimen of sulfapyridine in buffalo would be 104 mg/kg followed by 75 mg/kg at 24 h intervals.

Key words: Disposition kinetics, dosage regimen, sulfapyridine, buffalo

At present sulfapyridine, alone as well as in combination with trimethoprim, is widely used in the veterinary practice. In order to maintain the lowest plasma concentration in the course of therapy, an optimal dosage regimen of antimicrobial drug is necessary. The disposition kinetics and dosage regimen of sulfapyridine have been investigated in pigs (Vree et al., 1985), dogs (Scudi, 1940; Weber et al., 1943; Scudi and Childress, 1956) and rabbits (Smith and Williams, 1948) but such studies have completely been lacking in ruminants. The dosage regimen and disposition kinetics are calculated in animal species in which the drug is to be employed clinically (Nawaz, 1983). The present study was undertaken to investigate the disposition kinetics and suitable dosage regimen of sulfapyridine in buffalo.

Materials and methods

Animals and treatment. Experiments were performed on seven healthy male buffalo calves with body mass ranges of 159 to 222 kg. The animals were maintained under uniform standard conditions of the department and were supplied with green fodder of the season, wheat straw and water *ad libitum*. Sulfapyridine was administered at a single intravenous dose of 100 mg/kg body mass as a 33% solution in normal saline after dissolving it in a minimum volume of 4 N NaOH.

Blood samples were collected from the contralateral jugular vein into heparinized glass tubes at 1, 2.5, 5, 10, 15, 30, 45, 60, 90 min and 2, 3, 4, 5, 6, 7, 8, 10, 12, 24, 36 and 48 h after administration. Plasma was separated at room temperature after centrifugation at 3000 rpm for 15 min.

Experimental procedure. Sulfapyridine level was determined spectrophotometrically according to the method of Bratton and Marshall (1939). The concentration of N^4-acetylated derivatives of sulfapyridine was measured by the same method after acid hydrolysis. Free sulfapyridine levels in plasma were plotted on a semilogarithmic scale and analyzed by the method of Gibaldi and Perrier (1982).

Results and discussion

Fig. 1 shows the mean plasma concentrations of free sulfapyridine in buffalo calves following a single intravenous dose (100 mg/kg). At 1 min the plasma concentration was 512.5 $\pm$ 32.8 μg ml^{-1}, which rapidly declined to 275.9 $\pm$ 25.4 μg ml^{-1} at 15 min. Thereafter, the plasma levels of sulfapyridine gradually decreased to 18.5 $\pm$ 2.5 μg ml^{-1} at 48 h. Concentrations of more than 25 μg ml^{-1} were maintained from 1 min to 36 h after administration.

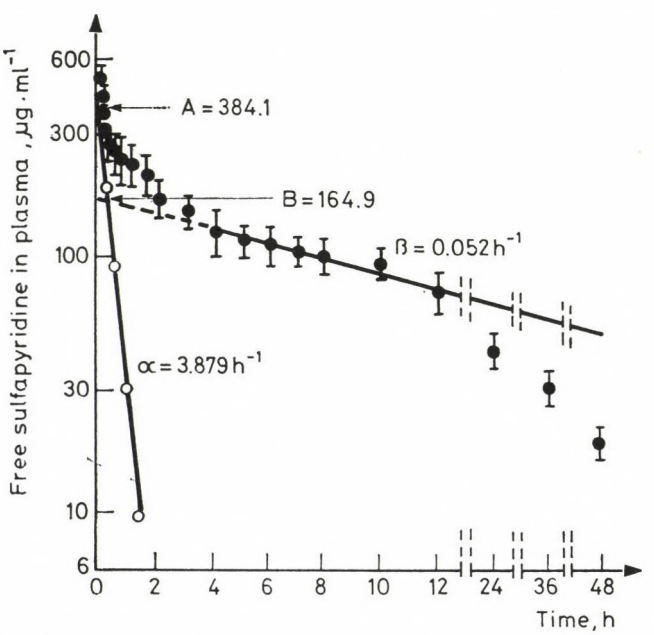

Fig. 1. Semilogarithmic plot of plasma concentration–time profile of sulfapyridine in buffalo calves following a single intravenous dose (100 mg/kg). Values are given as mean + SE of 7 animals. The data were analyzed by the biexponential equation $Cp = Ae^{-\alpha t} \pm Be^{-\beta t}$. Distribution ($\alpha$) and elimination ($\beta$) phase are shown by regression lines. The calculated points (O) of the distribution phases were obtained by the feathering technique

Table 1

Acetylation of sulfapyridine in the plasma of buffalo calves following a single intravenous administration (100 mg/kg)

Time	N^4-acetylated sulfapyridine (% of total plasma concentration)
15 min	3.45 ± 0.54
30 min	10.40 ± 2.11
60 min	9.80 ± 2.62
2 h	15.70 ± 0.31
4 h	12.50 ± 0.15
8 h	14.40 ± 1.62
12 h	13.90 ± 4.68
24 h	15.50 ± 3.14
36 h	16.90 ± 2.07
48 h	15.30 ± 1.45

Values given are mean ± SE of the results obtained for 6–7 animals

The data obtained for plasma levels of sulfapyridine were described by a two-compartment open model and the disposition curve was divided into distribution and elimination components. Other sulfonamides viz. sulfadimidine (Jha, 1983), sulfamethoxazole (Srivastava et al., 1987) and sulfaphenazole (Paul et al., 1977) have also been reported to follow the two-compartment open model in buffalo.

Sulfonamides are acetylated at the N^4-position by the enzyme system N-acetyltransferase and acetylcoenzyme A (Marshall et al., 1937). In the present study the N^4-acetylated form of sulfapyridine was found to be in the range of 3.45–16.9% at different time intervals (Table 1). No exact correlation, either between plasma concentration of drug and extent of acetylation or between time after drug injection and extent of acetylation, could be established, although for various sulfonamides acetylation has been reported to increase with time according to a hyperbolic pattern (Talseth and Landmark, 1977). In man (Vree et al., 1980) and rabbits (Vree et al., 1985) sulfapyridine is reported to be acetylated to the level of 15 and 50%, respectively.

The various disposition kinetic parameters of sulfapyridine are summarized in Table 2. From the high value of α (3.879 ± 0.164 h^{-1}) it is evident that sulfapyridine is rapidly distributed from the central to the peripheral compartment. Rapid distribution is further confirmed by the twofold higher value of K_{12} than K_{21}. The K_{12}/K_{21} ratio was calculated to be 2.15 ± 0.09. It is more useful to determine the extent of distribution of drug rather than the rate of distribution. The high values of Vd (area) (0.59 ± 0.03 Lkg^{-1}), T/P ratio (2.25 ± 0.09) and AUC (3314 ± 246 $\mu g\ ml^{-1}xh$) indicated that in buffalo sulfapyridine is rapidly and fairly distributed in various body fluids and tis-

Table 2

Kinetic determinants of sulfapyridine in buffalo calves following a single intravenous dose of 100 mg/kg

Parameter[a]	Unit	Range	Mean ± SE
CP°	$\mu g\ ml^{-1}$	498.9–643.1	549.1 ± 17.9
A	$\mu g_1\ ml^{-1}$	350.5–432.5	384.1 ± 11.3
α	h	3.174–4.516	3.879 ± 0.164
$t_{1/2\alpha}$	h	0.153–0.218	0.181 ± 0.008
B	$\mu g_1\ ml^{-1}$	138.8–210.6	164.9 ± 9.07
β	h	0.043–0.058	0.052 ± 0.002
$t_{1/2\beta}$	h	11.9–16.1	13.4 ± 0.52
K_{12}	h^{-1}	2.056–3.096	2.567 ± 0.134
K_{21}	h^{-1}	0.995–1.320	1.195 ± 0.045
K_{12}/K_{21}	Ratio	1.841–2.383	2.152 ± 0.094
Kel	h^{-1}	0.137–0.211	0.169 ± 0.009
$t_{1/2Kel}$	h	3.284–5.058	4.162 ± 0.223
AUC	$\mu g\ ml^{-1}\ h$	2492–4253	3314 ± 246
Vc	$L\ kg^{-1}$	0.15–0.20	0.18 ± 0.01
Vd (area)	$L\ kg^{-1}$	0.46–0.69	0.59 ± 0.03
Vd (B)	$L\ kg^{-1}$	0.47–0.72	0.61 ± 0.03
Vd (SS)	$L\ kg^{-1}$	0.43–0.67	0.58 ± 0.03
Cl_B	$ml\ kg^{-1}h^{-1}$	24.9–40.0	31.1 ± 2.28
f_c	Ratio	0.28–0.34	0.31 ± 0.01
T/P	Ratio	1.92–2.64	2.25 ± 0.09
Cp (24) (experimental)	$\mu g\ ml^{-1}$	38.4–47.6	42.1 ± 1.34
CP (24) (calculated)	$\mu g\ ml^{-1}$	34.5–60.0	47.9 ± 3.93
AT (24)	$\mu g\ ml^{-1}$	90.7–130.7	106.4 ± 6.69
DI (24)	$mg\ kg^{-1}$	52.4–104.3	75.2 ± 7.2
D	$mg\ kg^{-1}$	78.2–162.1	103.9 ± 11.6
τ	h	–	24 ± 0

The values given are from 7 animals.
[a] Kinetic parameters are described after Gibaldi and Perrier (1982)

sues. The identical values obtained for the volume of distribution (Vd) by various methods, viz. Vd (B), Vd (area) and Vd (ss), agree with Notari's (1973) conclusion that methods of calculation have no influence on the volume of distribution of the drug.

To evaluate the distribution of sulfapyridine, the drug levels (fractions of dose) in the central and the peripheral compartment were calculated and plotted on a semilogarithmic scale (Fig. 2). If a drug truly follows a two-compartment open model and injection was made rapidly by the intravenous route, the relationship of drug level between the central and the peripheral compartment at various time intervals can be framed by the following equation (Baggot, 1977):

Central compartment:

$$AC = \frac{Ao(\alpha\text{-}K_{21})}{\alpha\text{-}\beta} \cdot e^{-\alpha t} + \frac{Ao(K_{21}\text{-}\beta)}{\alpha\text{-}\beta} \cdot e^{-\beta t}$$

Peripheral (tissue) compartment:

$$AT = \frac{K_{12}Ao}{\beta\text{-}\alpha} .e^{-\alpha t} + \frac{K_{12}Ao}{\alpha\text{-}\beta} \cdot e^{-\beta t},$$

where Ao is the amount of drug in the central compartment at zero time, AC and AT are levels of drug in the central and the peripheral compartment, respectively, at time *t*. The results shown in Fig. 2 confirm the excellent distribution of sulfapyridine. The apparent equilibrium between plasma and tissues was achieved within 15 min. Once apparent equilibrium was attained, the drug concentration was always higher in the tissue compartment than in the central one.

The calculated elimination half-life of sulfapyridine in buffalo calves (13.4 ± 0.52 h) was identical to that found in pigs but considerably longer than that reported for dogs. The $t_{1/2\beta}$ of sulfapyridine in dogs (Scudi and Childress, 1956) and pigs (Vree et al., 1985) has been reported to be 5 and 12 h, respectively. In order to determine the mechanism of renal clearance of sulfapyridine in buffalo, the value of glomerular filtration rate (177.0 ml kg^{-1} h^{-1}),

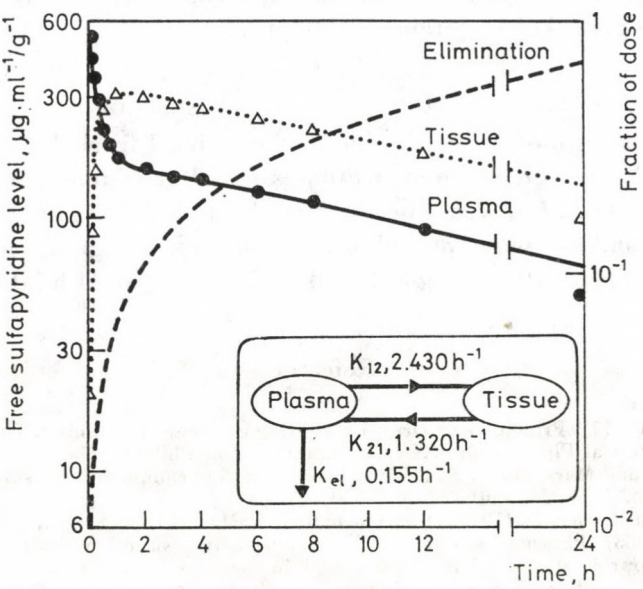

Fig. 2. Sulfapyridine level (fraction of dose) in the central and peripheral compartment and fraction of dose eliminated as a function of time in a representative animal following a single intravenous dose (100 mg/kg). A scheme of the two-compartment open model indicating values of first order rate constants in the same animal is also shown (in inset)

established by Varma (1980), was taken into account. The Cl_B of sulfapyridine was several times lower than the glomerular filtration rate. This indicates that the clearance of sulfapyridine in buffaloes may involve glomerular filtration as well as tubular reabsorption.

A satisfactory dosage regimen of sulfapyridine may be computed by employing the disposition kinetic parameters determined in the present study. Such a dosage regimen is only a guideline for judicious use, and the actual effectiveness of the drug still remains to be verified in clinical circumstances. For most of the sulfonamides the effective concentration, i.e. minimum inhibitory concentration (MIC), ranged from 0.16 to 64 $\mu g\ ml^{-1}$ (Mandel and Sande, 1985). In this calculation we have taken 50 $\mu g\ ml^{-1}$ as the MIC of sulfapyridine and this concentration is expected to be effective against all microorganisms which are effective to this sulfonamide.

The dosage interval based on the desired minimum plasma concentration is obtained by the following equation:

$$C_p\,(min)^{\alpha} = \frac{Be^{-\beta\tau}}{1 - e^{-\beta\tau}}$$

Fitting the values of B and β from Table 2 and the values of τ as 24 h, the plasma concentration between two successive dosings will be 65.1 $\mu g\ ml^{-1}$, which is more than our desired highest level of $C_p\ (min)^{\alpha}$ of sulfapyridine (64 $\mu g\ ml^{-1}$). Having chose a suitable and convenient dosage interval, the maintenance dose (D^1) is obtained as follows:

$$D^1 = C_p(min)^{\alpha}\ Vd.\ (e^{\beta\tau} - 1).$$

The priming dose (D) is obtained by omitting-1 from the above equation. Thus the priming and maintenance doses of sulfapyridine in buffalo, with a dosage interval of 24 h, would be 103.9 mg/kg and 75.2 mg/kg, respectively, or under field conditions the optimal intravenous dosage regimen of sulfapyridine in buffalo would be 104 mg/kg followed by 75 mg/kg at 24 h intervals.

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IMMUNIZATION OF DAY-OLD CHICKENS AGAINST NEWCASTLE DISEASE

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The avirulent Newcastle disease virus strain designated NDV-6/10, selected by B. Lomniczi at the Veterinary Medical Research Institute, Hungarian Academy of Sciences, is completely safe for day-old chickens by aerosol vaccination. Aerosol immunization using the Hungarian-made MASTERDROP® generator (particle size: maximum 7 μm) caused no vaccination reactions among 206,000 chickens with different maternal antibody levels. Other vaccines given simultaneously did not significantly affect the protection elicited against Newcastle disease (ND).

Almost 100% and 90% of the aerosolized chickens survived subcutaneous challenge with 10^6 LD_{50} NDV at 30 and 50 days old, respectively. A single immunization is sufficient for broilers; however, parent flocks should be revaccinated at 7 so 8 weeks old.

Key words: Newcastle disease, aerosol immunization, day-old chickens, avirulent strain NDV-6/10

Maternal antibodies to Newcastle disease virus (NDV) provide chickens with a relative protection that lasts up to 2–3 weeks of age. At the same time, their presence decreases the immunogenicity of live virus vaccines (Bennejean et al., 1978; Giambrone, 1981, 1985; Partadiredja et al., 1979; Robertson, 1981; Villegas et al., 1977). Maternal antibodies exert less expressed inhibitory effect on the growth of viruses administered in spray or aerosol vaccines onto the conjunctive or mucous membranes (Eidson and Kleven, 1976; Giambrone, 1985). Therefore, efforts have long been made to immunize day-old chicks against infectious bronchitis and Newcastle disease by this route (Van der Heide et al., 1957). The degree of protection elicited is inversely proportional to the particle size of the virus aerosol. However, too small droplets may cause vaccination reactions depending on the virulence of the vaccine strain and the health status of the flock (Giambrone, 1981, 1985).

In harmony with our experience gained in Hungary, several authors have reported such reactions for strain LaSota too. This is why clones of lower pathogenicity index have been selected from that strain (Eidson and Kleven, 1976, 1980). The moderate immunogenicity of live virus vaccines given at day old can be substantially enhanced by simultaneously giving a fraction of a

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dose of inactivated vaccine (Bennejean et al., 1978; Giambrone and Clay, 1986; Robertson, 1981).

As in Hungary there are hatcheries of enormous capacity, our aim was to find a method suitable for mass immunization of day-old chickens against ND without causing vaccination reactions.

Materials and methods

Chickens. The parent flock of the chickens immunized at 1 to 10 days old had been vaccinated twice with LaSota (in spray or via the drinking water) at grower age and with an inactivated oil-adjuvant vaccine before the laying season.

Virus strain. The vaccine strain was selected by B. Lomniczi at the Veterinary Medical Research Institute, Hungarian Academy of Sciences, from among numerous avirulent strains isolated in the field. The basis of selection was that this strain caused milder changes in chicken embryo kidney (CEK) cell cultures than did the known lentogenic strains. It did not kill embryos in 5 days, and even intracerebrally inoculated 1-day-old chicks remained healthy. Even doses 100 times the immunizing dose failed to cause vaccination reactions by any route of application. The strain is extremely heat stable. It was designated and patented as NDV-6/10.

Aerosol generator. The atomizing performance of the Hungarian MASTERDROP (MD-2500)® apparatus (patented by Tamási et al.) is 2.5 l/h. From the atomizer the aerosol gets into a separating space from where droplets larger than 7 μm flow back into the tank. The aerosol stabilizer used (DORY′ DROPS)® substantially hinders evaporation for 25 to 30 min. One generator is needed for every 500 m^3 air-space. The virus dose used is $10^{7.3}$ EID_{50} per cubic metre of air. The chickens stay in the virus aerosol for 30 min.

Haemagglutination inhibition (HI) test. The HI test was done in the usual way, using 4 HA units of strain LaSota. The geometric mean titres were expressed in $\log_2$ values.

Challenge. 10^6 LD_{50} of a mixture of virulent virus strains, used by the State Institute for the Control of Veterinary Vaccines and Pharmaceuticals for several decades, was injected subcutaneously in 0.1 ml of allantois suspension. All susceptible control birds died on days 5—6.

Results

The results of experiments aimed at determining the optimum immunizing dose of virus are not given here in detail. Chickens that had been exposed to $10^{6.0}$ EID_{50}/m^3 virus by inhalation possessed 90 and 100% protection

against challenge at 4 and 7 weeks old, respectively. Chickens immunized with $10^{7.0}$ EID_{50}/m^3 virus showed 100% survival rate upon challenge both at 4 and at 7 weeks old. As in the practice the air-space of the chicken houses cannot be isolated hermetically, under field conditions we used virus doses of $10^{7.3}$ to $10^{7.5}$ EID_{50}/m^3 for immunization.

Immunization of broilers

Up to 1990, a total of 86,050/broiler chickens of 5 flocks were immunized. The results are summarized in Table 1. In the first field trials, chickens immunized at 1 day old (in the hatchery or in the chicken house) were challenged at different ages. If the chickens were immunized only against Newcastle disease with the NDV-6/10 vaccine, at 30 days old they showed 98 to 100% protection. If the aerosol contained, besides our NDV-6/10 strain, also strain H_{120} (INTERVET®) of infectious bronchitis virus (IBV), almost 92% of the chickens survived challenge at 1 month old. The group immunized only against ND showed 90 to 94% protection at 50 to 54 days old, while that immunized with NDV-6/10 plus IBV strain H_{120} (INTERVET®) possessed 75% protection at 50 days old.

Chickens of a large broiler flock numbering almost 40,000 birds, immunized at 9 days old, showed 100% protection at 44 days of age (at slaughter). The body mass at slaughter of chickens of that group, stocked at a higher rate than usual, was 1530 g and their mortality rate 3%.

In another flock of 40,000 birds, immunized against ND with the NDV-6/10 vaccine at 3 days old and later affected by mycoplasmosis, the rate of protection was 91% upon challenge at 33 days old.

Table 1

Immunization of broilers with NDV-6/10 aerosol ($10^{7.3}$ EID_{50}/m^3)

Number of vaccinated chickens	Age of chickens (days)		Died/Infected	Survival rate
	at vaccination	at challenge		
500	1	14	0/20	100.0
		28	0/20	100.0
		54	1/10	90.0
850	1	30	2/50	98.0
		50	4/75	94.7
5,100+	1	30	5/60	91.7
		50	5/20	75.0
39,600	9	44	0/30	100.0
40,000++	3	33	5/55	91.0

+ the birds simultaneously received H_{120} aerosol; ++severe mycoplasmosis

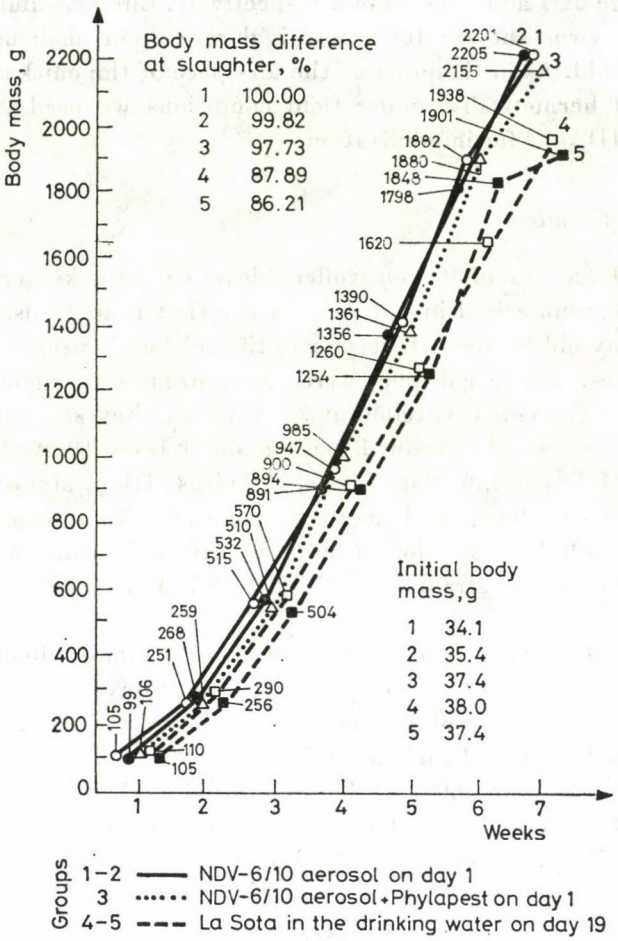

Fig. 1. **Body mass gain of chickens immunized in different ways (on the basis of weekly averages)**

In the immunized broiler flocks neither depression nor respiratory signs were seen after vaccination. The mortality rate was substantially lower than had been typical of that farm earlier. The steady body mass gain shown in Fig. 1 deserves particular attention. Comparing the body mass gain of the 5 experimental groups kept under indentical conditions, it can be seen that groups 1 and 2 (groups that had been exposed at day-old exclusively to an aerosol containing our experimental NDV-6/10 strain) showed the steadiest body mass gain and reached the highest body mass. If the birds simultaneously received an inactivated vaccine (Phylapest) as well, their body mass gain was slightly lower. Birds of groups 4 and 5, given LaSota virus via the drinking water at

19 days old, reached a 14 to 17% lower body mass than did those of groups 1 and 2 kept under the same conditions. Clinical signs were not seen in chickens of groups 4 and 5 either.

Immunization of parent flock and laying flocks producing for the market

Up to 1990, a total of 120,370 birds of 4 large-scale flocks, immunized against Marek's disease in the hatchery, were used. The data are summarized in Table 2. Chickens of flocks II and III had also been immunized with IBV strain H_{120} (INTERVET®) in the hatchery.

Birds of the four flocks were immunized with an aerosol of our new NDV-6/10 strain at 2 to 10 days old upon arrival at the receiving farm. The times of immunization against infectious bronchitis (IB) and infectious bursal disease (IBD) are shown in the table. Challenges performed between day 30 and 65 of life revealed 83 to 100% protection. In the first two months, the mortality rate remained below 3% in the immunized flocks, the only exception being the cockerel stock in group A of flock 3 where it was 5.17%.

Table 2

Immunization of the parent flock and laying flocks

Number of vaccinated chickens	Age at vaccination and route of vaccination	Age at challenge (day)	Died/Infected	Survival rate (%)
Flock I				
32,836	Day 1: Marek sc.			
	Days 2–4: NDV–6/10 aerosol	30	0/60	100.0
	Day 35: IBV (H_{120}), drinking water	60	10/61	83.6
Flock II				
28,204	Day 1: Marek sc. IBV (H_{120}) spray			
	Days 2–10: NDV–6/10 aerosol	45–53	2/52	96.1
	Days 27–35: IBDV			
	Days 34–42. IBV (H_{120}) drinking water			
Flock III				
30,080	Day 1: Marek sc.	36–40	A 0/20	100.0
	IBV (H_{120}) spray		B 0/20	100.0
			C 0/20	100.0
	Days 4–8: NDV–6/10 aerosol			
	Day 24: clone 30 (group C)	59–63	A 1/20	95.0
			B 0/20	100.0
			C 0/20	100.0
Flock IV				
29,250	Day 1: Marek sc.			
	Days 5–8: NDV–6/10 aerosol	62–65	2/40	95.0
	Days 22–25: IBV (H_{120}) drinking water			

Around the age of 2 months, these flocks were again immunized with an aerosol containing our test vaccine strain. In laying flocks producing for the market the third vaccination with our test vaccine was carried out also by aerosolization, before the beginning of the laying season. Experiments are currently under way to determine how many times and when aerosol immunization should be repeated during the laying season.

In breeding flocks, aerosol immunizations in the first week of life and at 2 months old were followed by a trivalent (NDV–IBV–IBDV) inactivated, oil-adjuvant vaccine (INTERVET®) which elicited an expressed booster effect and high HI titres.

Discussion

The extensive trials, mostly conducted in large flocks, have proved the safety of our test strain. In the flocks immunized with this strain, mortality was lower and the body mass gain of broilers substantially higher than the local values obtained earlier. Simultaneous immunization against Marek's disease and IB did not appreciably decrease the high level of protection achieved against ND. The same was found 2–3 weeks after vaccination of growing chickens against IBD. This observation is in harmony with the findings of other authors (Giambrone, 1981; Giambrone and Clay, 1986; Robertson, 1981).

An important experience is that aerosols of very small droplet size confer adequate protection in spite of the maternally derived immunity. For lack of space, the dynamics of antibody responses is not given here in detail. Antibody response was protracted: maximum titres were demonstrable only around day 30. In agreement with the observations of others (Bennejean et al., 1978; Eidson and Kleven, 1976, 1980; Giambrone, 1985; Robertson, 1981; Villegas et al., 1977; Yadin, 1976), we also found a few seronegative birds. It was this group which yielded the birds that succumbed to challenge; however, most of these birds survived the infection. Similar observations have been reported by others (Giambrone, 1985; Holmes, 1979; Parivallal et al., 1986; Partadiredja et al., 1979; Villegas et al., 1977) as well. All this should be taken into consideration when evaluating the HI test of chickens immunized at an early age. The slight difference between results of the HI test performed with strain LaSota and with the homologous antigen was similar to what was found by others for HI tests with the conventional strains and the new isolates (Alexander and Parson, 1986).

Because of the risk of vaccination reactions, aerosols of less than 10 μm droplet size are rather rarely used for vaccination against Newcastle disease (Giambrone, 1985). Our preliminary studies suggest that the safety of strain NDV-6/10 has a decisive role in the absence of vaccination reactions. Further

studies have led to registration of the vaccine produced from strain NDV-6/10 in Hungary by the name of VITAPEST and its commercial availability. These experiments will be reported later.

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BOOK REVIEWS

Wolfgang LÖSCHER, Fritz Rupert UNGEMACH and Reinhard KROKER: *Grundlagen der Pharmakotherapie bei Haus- und Nutztieren.* First edition. Verlag Paul Parey, Berlin und Hamburg, 1991. 380 pages, 59 tables and 12 figures. ISBN 3-489-57416-8.

The book, which summarizes the most important knowledge available on the pharmacotherapy of domestic and farm animals, consists of three parts: a general introduction, specific pharmacology and pharmacotherapy, and the appendix.

The *general part* deals with the fundamental elements and specific areas of pharmacology, the definitions and legal provisions concerning pharmaceuticals. The aspects and order of describing individual drugs and the literature used in writing the book are summarized here.

The most spacious part of the book is the second part, *special (detailed) pharmacology and pharmacotherapy*. It describes the different groups of pharmaceuticals in 21 chapters, on about 320 pages. The pharmaceuticals are categorized by their pharmacodynamic properties and are discussed with an up-to-date attitude, relying upon the most recent data.

Each chapter is preceded by a well-systematized and comprehensive introduction which, building upon physiological and biochemical bases, makes the reader understand the essence of pharmacodynamics on the molecular level. Within each chapter, the pharmaceuticals are grouped and described in a very logical manner. Simplified and highly illustrative figures help the reader understand the complex pharmacodynamic relationships and the text is well complemented with tables which supply factual data.

The individual chapters can be briefly characterized as follows.

The book discusses the drugs acting on the *nervous system* in four distinct groups: pharmaceuticals acting on the autonomous nervous system, those affecting peripheral mediators, drugs acting on the central nervous system, and local anaesthetics. Assigning the peripheral mediators to a distinct group can be considered a relatively new approach. In this group are described histamine, 5-hydroxytryptamine (serotonin), and the prostaglandins.

In the chapter devoted to *cardiac drugs*, besides the classic cardiac glycosides the authors devote rather large space to antiarrhythmic preparations which have at present mainly human therapeutic significance in Hungary. This part mentions also the newest preparations. Because of the relative scarcity of data available for domestic animals, it must have been difficult to point out the species specificities and, with the exception of the dog, to give the accurate dose regimen. The same statements apply to the *drugs acting on circulation* comprising hypertensive and hypotensive drugs.

The chapter dealing with *infusion therapy* affecting water and electrolyte balance is a well-arranged part of the book which contains the most recent concepts and body of knowledge. Besides various parenterally applicable electrolyte solutions, oral rehydration, correction of acid-base imbalances, potassium substitution, and solutions containing calcium and magnesium are also described, together with carbohydrate-containing solutions and preparations suitable for plasma substitution.

A concise, well-arranged chapter is devoted to drugs acting on *renal function* (diuretics and antidiuretics). Pharmaceuticals influencing *uterine function* are described only sketchily. Considering their importance in the veterinary practice, these drugs could perhaps be discussed in more detail.

The chapter about drugs acting on the *respiratory system* is very successfully written and comprises the most recent preparations that can be used also in veterinary medicine. The description of pharmaceuticals serving for the treatment of *hepatopathies* is practical but too

concise. Drugs acting on the liver, the central organ of metabolism, should have deserved a somewhat more detailed description.

The description of drugs acting on the *gastrointestinal tract* (antacids, antazymotics, emetics, antiemetics, antidiarrhoeals) is very successful in its contents, proportions and up-to-dateness alike.

The chapter dealing with *disinfectants* is rather short and describes the preparations only sketchily, though it offers a practical grouping and a good overall view.

The drugs suitable for *treating and preventing bacterial infections* (antibiotics and other chemotherapeutics) are described on 35 pages. This is an especially well-written and clearly arranged chapter in which reference is made to new drugs which are little, or not at all, used in veterinary medicine yet. These drugs mark out the new directions of development.

Corresponding to their significance, *antiparasitic drugs* are dealt with in almost as much detail as antibacterial preparations. Separate chapters are devoted to anthelmintics and drugs against ectoparasites. Practically all important preparations can be found in the book. Drugs of natural origin and exclusively synthetically derived ones are distinguished.

A well-arranged description of *antimycotic preparations*, both classical and recently developed, can also be found in the book.

Due to their limited veterinary medical importance, *cytostatic agents* are dealt with very briefly (in 2.5 pages). Beyond the general principles, a table is presented and dose regimens of some preparations for different animal species are given.

Vitamins and *trace elements* are described in the same chapter according to the classical grouping. The chapter dealing with *hormones* and *drugs of hormonal effect* is also very well written and contains the most recent therapeutic principles and preparations. Somatotropins developed as a result of biotechnological research are also described in this chapter. No separate chapter is devoted to growth promotants.

In relation to the size of the book, the pharmacological influencing of *inflammation* has received rather great emphasis (30 pages). Besides the well-known antiphlogistics (non-steroid antiinflammatory drugs and corticosteroids), this part describes dimethylsulfoxide, Orgotein, and the substrates of articular metabolism as well.

The last chapter is devoted to the therapy of important *toxicoses*. Besides nonspecific descriptions, the main emphasis is given to specific antidotes and procedures. Such rarely used compounds as D-penicillinamine and iron(III)hexacyanoferrate(II) can also be found in this well-written chapter.

The *Appendix* deals with the conversion of human drug doses to animals, drug combinations, the use of approved pharmaceuticals on fish, drugs approved for use in poultry (summarized in a table), the drug treatment of laboratory animals and pets, medicated diets, treatment of protozoal diseases and the undesirable risks associated with the use of pharmaceuticals.

This book is an excellent work comprising large areas of veterinary pharmacology. It gives evidence of its authors' wide-ranging professional knowledge extending from molecular pharmacology to the practical application of drugs. Although the book is intended for veterinarians, it will prove useful also for specialists of other related fields (pharmaceutists, researchers of experimental medicine and life sciences) as well as for university students in these fields. The readers get abundant information in a well-systematized, concise form.

The work contains, mostly in systematic order, also partial data which are hard to obtain from most of the currently available text-books (e.g. pharmacokinetic data for domestic animals, accurate dosing, biological utilization, contraindications, undesirable side effects and interactions). From the food sanitary point of view it is of prime importance that drug withdrawal times are given accurately.

As the title of the book indicates, the authors wished to summarize the fundamentals of the pharmacotherapy of domestic and farm animals. By expanding some of its chapters, this excellent work could be developed into a high-standard text-book or manual in the future.

Ferenc SIMON

H. SOMMER, E. GREUEL and W. MÜLLER: *Hygiene der Rinder- und Schweineproduktion.* Second, revised edition. Eugen Ulmer Verlag, Stuttgart, 1991. 568 pages, 91 figures, 250 tables.

Since the first edition published in 1976, thorough changes have occurred in the economy of the former Federal Republic of Germany. As compared to the 1970's, completely new diseases and health problems have come to the fore in animal production. In an attempt to conform

to these changes, the authors have substantially revised and, in places, enlarged the first edition, laying great stress on veterinary prevention.

The first chapter, entitled "*General hygiene*" (115 pages), was written by *W. Müller*. The chapter is subdivided into several parts. The first part is entitled "*Factors affecting health*". In it the author divides the environmental factors acting on animals into living (bacteria, viruses, fungi and yeasts, endo- and ectoparasites) and non-living (inanimate) factors (microclimate of animal houses), and discusses them in detail in that grouping. The description of diseases of cattle and swine is abundantly illustrated with tables. The pages dealing with water, soil and air hygiene form a valuable element of this part. The second part bears the title "*General pathology*". It describes in detail the role of constitution and disposition in the development of diseases. The author illustrates the galenic signs of inflammation, the degeneration and necrosis of organs and tissues, and the pathology of the circulatory system by examples. The third part, entitled "*Defensive mechanisms of animals*". offers a clear and comprehensive overview of the related body of knowledge. The fourth part, which bears the title "*Animal production and the veterinarian's tasks*", has been written almost entirely with a view to prevention. It describes the examination of the drinking water, the microclimate of animal houses and the feed, faecal examination for parasites, and disinfection procedures.

The second chapter of 158 pages, entitled "*Specific hygiene of cattle production*", was written by *H. Sommer*. The part entitled "*Dairy cows*" first discusses the economic importance of cattle production, then gives the main statistical data indicative of production. In 1987, 49.6% of all dairy cows of the FRG were kept on farms with a capacity for 20—49 cows and only 0.9% of them were kept on farms holding more than 100 cows. The change in milk production was in a positive correlation with the incidence of reproductive disturbances, foot problems and mastitis. The author devotes much space to the control of noninfectious diseases and the possibilities of reducing the losses cansed by them. Ecto- and endoparasite control and the measures to be taken against mastitis are also described here. It is mentioned that ninety per cent of subclinical mastitis cases are caused by cocci. The phases of the professional milking procedure and the care and maintenance of milking machines are summarized in tables. The parameters of bovine blood are given to facilitate evaluation of blood samples submitted to the laboratory. The part "*Calves and growing cattle*" is also subdivided. The author suggests that farmers shall always have in reserve deepfrozen colostrum from older cows, so that newborn calves can be properly attended to also if their dams suffer from agalactia or mastitis or if they die. He analyses the main sources of losses in the prenatal, perinatal and postnatal period, and outlines the infectious and noninfectious diseases of growing calves. This part is complemented with carefully arranged and compiled tables. Only 3% of the farmers make use of a special extension service: on their farms the calf losses are around 3%. The aetiology, clinical features and therapy of digestive and respiratory diseases are dealt with in much detail. The table showing the clinical signs, names and causative agents of calf diseases grouped by age is a valuable aid in systematizing the available knowledge.

The chapter entitled "*Specific hygiene of swine production*" (103 pages) is also the work of *H. Sommer*. First it deals with the economic importance and structure of swine production. It demonstrates the process of concentration with concrete data: while in 1975 the proportion of farms of at least 50 sows was 14%, by 1988 this ratio rose to 48%. During the same period of time, the number of farms holding at least 400 fattening pigs increased threefold. The author suggests that nonpregnant ("open") sows should be kept on bedding. Nursing sows are less and less kept in individual pens, tied down with a collar. Comprehensive tables help in distinguishing between noninfectious and infectious diseases, and the author follows the same approach in the case of ecto- and endoparasites. A separate part is devoted to reproductive disturbances which are especially severe in places where the keeping of sows does not correspond to their behavioural properties. The part entitled "*Piglets and growing pigs*" first deals with the different management practices. Perinatal and postnatal losses are smaller if the piglets are kept on bedding. The infectious and noninfectious diseases of piglets and growing pigs are outlined with the help of tables. The chapter is concluded by a comprehensive table which presents the names, clinical signs and causative agents of pig diseases by age group and refers the readers to specific pages of the book for more details.

The chapter entitled "*Veterinary hygiene*" (142 pages) was written by *E. Greuel*. The first part of the chapter outlines the administrative tasks associated with the control of disease outbreaks. The legal provisions concerning this activity are specified in the Animal Disease Outbreaks Act. The most important element of prevention is obligatory notification: this applies not only to disease outbreaks but also to the suspicion of diseases. Notifiable epizootic diseases of cattle and swine and animal diseases falling under obligatory notification are listed in tables. The second part, entitled "*Zoonoses*", also sums up the most important diseases in tables. The hygienic regulations specified for the destruction and disposal of animal carcases

are also discussed here. In the part entitled "*Regulations concerning the purchase of animals*" several examples are presented to demonstrate how to make contracts. Part 4, entitled "*Animal protection*", offers a complete description of the animal protection act currently in force, complemented with explanations and addenda. The details of "*Regulations concerning pharmaceuticals*" are described in a separate chapter. The conditions of using drugs in concentrated animal keeping deserve particular attention. Part 6 tackles the problem of "*Meat-hygienic regulations*" in great detail. The parts entitled "*Milk-hygienic regulations*" and "*Food-hygienic regulations*" are also circumspectly written.

A long reference list, grouped by chapter, is given at the end of the book. This list will be a great help also to those interested in the details.

This scientific work will be helpful also for specialists working in the practice. The large number of well-arranged tables is a great advantage of the book, together with the decimal classification used in the text. It is also remarkable that the authors frequently mention the financial aspects of the veterinary activity. The excellent typographical make-up of the book praises the publisher, Eugen Ulmer Verlag.

Alajos BALLÁSCH

Imogen RÜSSE and Fred SINOWATZ: *Lehrbuch der Embryologie der Haustiere.* Verlag Paul Parey, Berlin and Hamburg, 1991. 473 pages, 318 photographs, 710 figures (of them 83 multicoloured) and 39 tables. Price: 178.– DM.

The new text-book of embryology of domestic animals is a successor of Zietschmann and Krölling's "Ontogeny of Domestic Animals". It was written by Prof. Dr. Imogen Rüsse and Prof. Dr. Fred Sinowatz (Institute of Veterinary Anatomy, Munich), with the collaboration of Prof. Dr. Angela von den Driesch (Institute of Paleoanatomy, Domestication Research and Veterinary History, Munich). The drawings were made by Bettina Buresch and Barbara Ruppel (Munich).

The new text-book summarizes the embryonic and fetal development of cattle, horses, pigs, small ruminants, dogs, cats and birds. It illustrates the different stages of ontogeny (from fertilization of the ovum up to birth) with detailed tables. The book devotes particular attention to early embryonic development and, thus, it will be an important aid in carrying out new biotechnical procedures in domestic animals. Matterful chapters deal with gametogenesis, the sexual cycle of female animals, the fertilization and segmentation of the ovum, early pregnancy, implantation, and the development of fetal membranes. The part dealing with the biology of reproduction seems to be the most valuable chapter of the book and contains the greatest amount of new information. Further parts deal with the ontogeny of the nervous system and the urogenital organs. The development of the facial region is demonstrated on the example of the sheep and the cat. The work reflects both the authors' own research results and Hungarian as well as foreign data of the literature relating to the subject.

Contents. History of embryology of domestic animals. *General embryology:* General principles of ontogeny and morphogenesis. Gametogenesis. The sexual cycle of female animals. Fertilization. Segmentation and division. Gastrulation, formation of germinal epithelium, and development of the fetal body. Early pregnancy, implantation and development of fetal membranes. *Specific embryology:* The heart and the circulatory system. The nervous system. The organs of sense. The urogenital organs. The digestive tract and its accessory organs. The respiratory organs. Body cavities. The organs of locomotion. The skin and its appendages. Face and body shape. Development of abnormities. Subject index.

It is well known that the subject-matter of embryology can be found rather dispersedly in the special literature. This makes the authors' laboursome synthesizing and analytical work especially valuable. The detailed list of selected references given at the end of each chapter is a substantial aid to specialists who wish to gather information quickly. The book is abundantly illustrated with original drawings and photographs taken of preparations at the Institute of Veterinary Anatomy, Munich. The high-quality drawings and photographs reflecting the use of modern phototechnical procedures are a valuable asset to the book.

This up-to-date and high-standard text-book and manual will be an indispensable aid to students of veterinary medicine, veterinarians, zootechnicians, physicians, biologists and embryologist. We may recommend it with perfect confidence for all colleagues and also for teachers and researchers working in "borderline" areas of veterinary science.

László ZÖLDÁG

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DIETARY FACTORS INFLUENCING PROTEIN UTILIZATION: A REVIEW

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(Received December 15, 1991)

Utilization of dietary protein depends not only on the amino acid composition and the digestibility of the protein itself, but also upon the level of protein, feed intake, energy, fibre, and other constituents in the feed. On the other hand, the animal species, sex, age and health status may also play an important role. A deeper understanding of these factors helps to utilize feedstuffs as a protein source more efficiently.

Keywords: Protein utilization digestibility dietary factors, role of species

The nutritive value of a feed protein depends primarily on its capacity to satisfy the needs of animals for amino acids. Different protein sources may have different nutritive values depending on their ability to provide essential amino acids for the various biochemical functions of the organism. As the essential amino acid requirements may vary according to the age and physiological status of the animal (maintenance, growth, pregnancy, lactation, etc.) and the type of production (meat, milk, egg, etc.), the same protein consequently may have different nutritive values, depending on the criteria used for its evaluation.

The protein quality of dietary proteins is primarily determined by the content and proportion of their available amino acids. However, the nutritive value of a feed or feed ingredient is not independent of the animal to which it is fed. The composition and adequacy of the diet as a whole and the physiological, nutritional and health status of the animal influence the capacity of the feed protein to meet the amino acid requirement of the animal.

Levels of dietary protein, energy, fibre, tannin, the so-called antinutritive substances as well as environmental circumstances (housing, temperature, light programme, etc.) and the feeding techniques all may influence protein utilization to a different extent. Species, strain, age, sex, health and physiological status also may have an effect on protein digestion and absorption. In bioassays for evaluation of the protein quality these factors are to be held more or less constant to get reliable results depending primarily on the digestibility and amino acids composition of the raw protein tested.

This review gives a short overview of the most important factors which are independent of amino acid composition of the feed protein and may influence protein utilization.

Dietary protein level

The growth performance of animals improves with increasing dietary protein levels within certain limits according to a saturation model. The efficiency of protein utilization, however, gradually falls.

Forbes et al. (1958) demonstrated in rat experiments that *biological value* (BV) declines with increasing protein levels. BV was found to be sensitive to changes in dietary protein concentrations (Eggum, 1973); however, there seemed no fixed relation between crude protein levels and BV, depending on the quality of dietary protein. BV may increase at increasing crude protein levels in a low dietary protein concentration range (60–80 g CP/kg DM), but it declines at levels higher than about 160 g CP/kg DM at an increasing rate (Fig. 1). *True digestibility* values for rats were independent of dietary protein levels of 60–225 g CP/kg DM, however they declined at higher protein levels (Eggum et al., 1986). At the same time, the *apparent digestibility* values were shown to increase in a curvilinear manner, reaching a plateau (Fig. 2).

The *net protein utilization* (NPU) values are also negatively affected by increasing dietary protein levels. Other indices of protein value (Protein Efficiency Ratio = PER, Net Protein Ratio = NPR, Liver Protein Utilization = LPU) determined by rats were also shown to decrease if dietary protein concentration increased (Krajcovicova and Dibak, 1980) as illustrated in Fig. 3.

Net protein utilization values of extracted soybean meal fed to growing rats as sole source of protein decreased gradually (79.2, 69.9, 59.0, 53.6) when dietary protein levels were increased from 10% to 14, 18 and 22% respectively (Hegedűs et al., 1989).

The level of dietary protein may affect the turnover rate of tissue proteins. With increasing dietary protein levels the muscle and liver protein degradation decreases (Hayase et al., 1984). The lower protein turnover rate may explain the lower efficiency of protein utilization at higher dietary protein levels.

Level of feed intake

The negative effect of increasing dietary protein levels on the efficiency of protein utilization is well documented. However, in pigs increasing levels of daily feed intake may have a positive influence on efficiency (NPU) if higher dietary protein levels are used (Fuller and Crofts, 1977). An explanation for this is that the low efficiency at higher dietary protein levels may be improved by the extra energy supplied by the greater amount of daily feed ration.

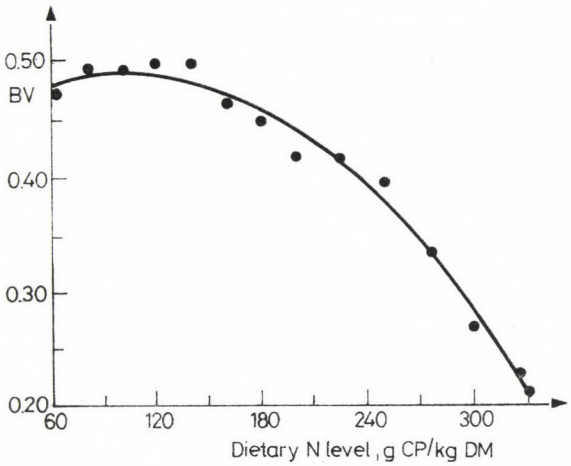

Fig. 1. Changes in biological value (BV) with increasing dietary levels of nitrogen in the rat (Eggum et al., 1986)

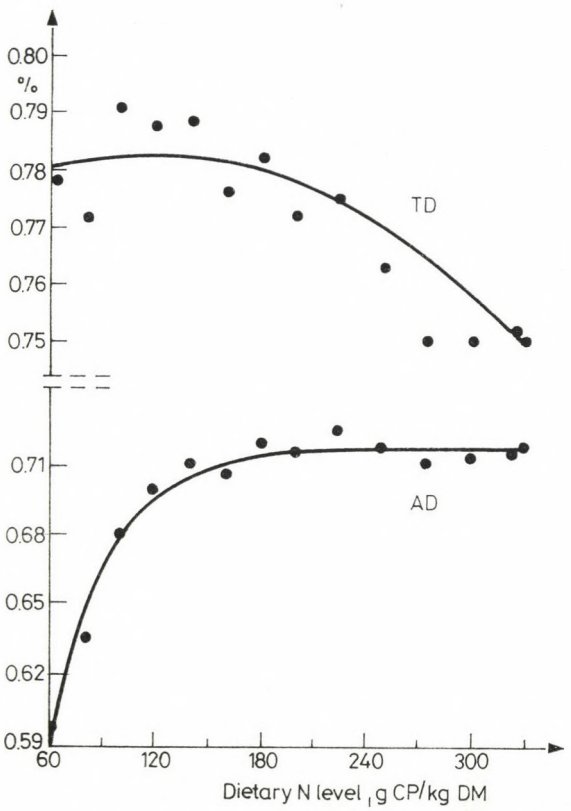

Fig. 2. Changes in true (TD) and apparent (AD) protein digestibility with increasing dietary levels of protein in the rat (Eggum et al., 1986)

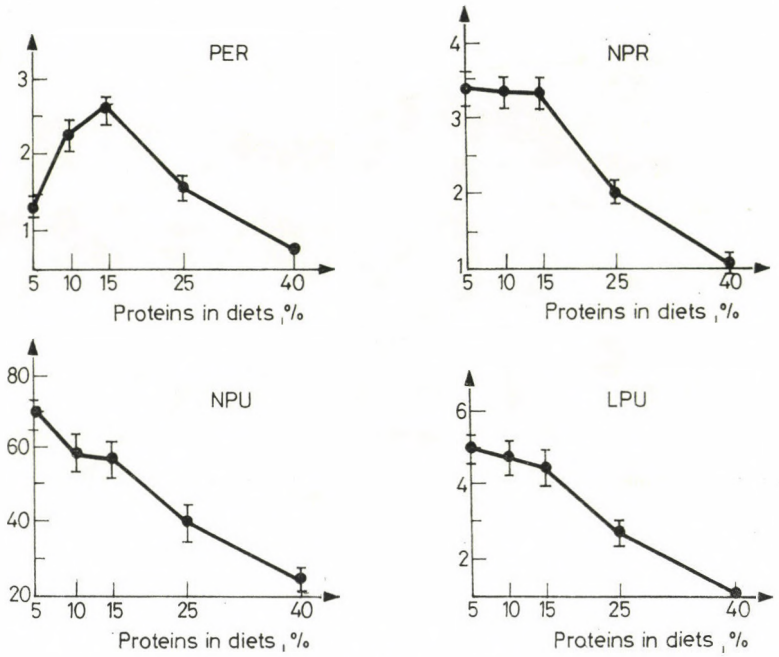

Fig. 3. Changes in protein efficiency ratio (PER), net protein utilization (NPU), net protein ratio (NPR) and liver protein utilization (LPU) with increasing dietary levels of protein in the rat (Krajcovicova and Dibak, 1980)

Ad libitum or restricted feeding of pigs did not seem to greatly affect apparent nutrient digestibility measured at the end of the small intestine (ileal digestibility; Jorgensen et al., 1981); however, apparent digestibility values measured over the entire tract (faecal digestibility) were slightly increased if feeding level was restricted (Fekete and Gippert, 1981; Haydon et al., 1984). The level of feed intake may have only a minor effect on the efficiency of protein utilization under the conditions of animal bioassays such as BV, NPU and NPR.

The addition of water to the dry rations has no beneficial effect on protein utilization (Dreyer, 1979).

Dietary energy level

The effect of total energy intake on protein utilization may differ depending on whether the supply of energy is limiting or is above the requirement. Protein accretion requires energy. The total cost of protein deposition is about 1.6–3.1 KJ ME/KJ protein, i.e. 38–74 KJ/g protein (Eggum et al., 1980; Müller and Kirchgessner, 1979). Dietary fats and carbohydrates as

energy-yielding nutrients may stimulate protein deposition, which is described as a "protein-sparing" effect (Munro, 1964). Dietary carbohydrates moreover may enhance the rate of release of insulin, which stimulates muscle protein synthesis and decreases protein catabolism (Fuller et al., 1977).

Under the conditions of practical animal feeding an increased protein intake may not be fully effective if dietary energy is limited. High dietary energy supply may have a positive effect on protein utilization only if higher than 10 per cent dietary protein levels are used. At lower protein levels, commonly used in animal assays for BV or NPU, the relatively high energy content of the diets had only limited effect on protein utilization irrespective of the quality of protein source in the diet (Eggum, 1978).

Fat enrichment of the diets has no heavy influence on protein digestibility, although the slight improvement on protein retention using low dietary protein levels, as a consequence of high dietary fat supply, may be explained by the deposition of collagen in subcutaneous fat tissues, thus improving the retention of dispensable amino acids from the diet (Szelényi-Galántai et al., 1980).

The reduction of energy intake causes endogenous nitrogen output to rise in asymptotic fashion, however the utilization of protein as a source of energy is very limited below 10% dietary protein level. At higher dietary protein levels protein utilization may be impaired only if energy supply is restricted more than about 50 per cent of the requirement (Dreyer, 1979).

Dietary fibre level

An increase in crude fibre in diets may have a negative effect on apparent or true digestibility. Fibre from native sources (husk from sunflower, straw, etc.) may increase endogenous faecal nitrogen excretion (Meier and Poppe, 1977; Bergner et al., 1980) and it can also adsorb amino acids liberated during proteolytic digestion (Howard et al., 1986). Moreover, proteins and amino acids may be located unavailably within intact cell walls (Meier and Poppe, 1977). However, purified fibre sources (e.g. cellulose powder) have only a minor influence on digestibility (Sauer et al., 1979; Eggum, 1973).

The water-holding capacity and the abrasive effect on the intestinal wall of dietary fibre depends on its physical and chemical characteristics. The lower protein digestibility associated with some hull fraction (canola, soybean) may be proportional to the lignin and tannin rather than the cellulose content of the fibre source (Mitaru and Blair, 1984; Gagne and Acton, 1983).

The source and level of fibre in experimental diets may negatively affect protein retention by reducing the intestinal activities of digestive enzymes (Boisen et al., 1985), by stimulating microbial activity in the digestive tract

and by influencing the level of feed (protein) intake (Eggum et al., 1982, 1984, 1986; Eyre, 1985).

Protein utilization in pigs may show an optimum with increasing dietary fibre concentration within certain limits, whereas apparent digestibility proportionately decreases with it (Fekete, 1979).

The efficiency of protein utilization may depend on the food passage time. A small amount of pectin (3%) instead of the conventional cellulose increases gastric emptying time. This observation (Muhs et al., 1987) may be useful in formulating experimental (sliming) diets for delayed gastric emptying.

Tannin content of the diet

Tannins are polyphenolic compounds which can form insoluble complexes with proteins. They occur in a wide variety of plants used for feeds and feeds including beans, barley, millet, sorghum, legumes, cider wine, tea, etc. The most apparent nutritional effects of tannins are depression of the growth rate, reduced feed efficiency, poorer protein digestibility and utilization, as documented in rats and chicks. The situation with pigs is less clear. The microflora of the alimentary tract of animals adapted to plant diets (ruminants, horses, rabbits) is able to greatly reduce the toxicity of orally administered phenolic compounds. Tannins can have also a negative effect on feed intake. The influence of tannin on protein utilization has been comprehensively reviewed by Eggum and Christensen (1975).

Microbial activity in the digestive tract

The presence of microorganisms in the alimentary tract contributes to digestion. The extent of digestion, however, depends on the composition of the microflora and the quantity of undigested feed residues. Undigested materials (fibre, starch, proteins, etc.) stimulate bacterial growth in the hindgut. During microbial fermentation in the caecum and the large intestine, amino acids may both be deaminated and synthesized and volatile fatty acids are also produced. The absorption of the products of microbial digestion (fermentation) may contribute to the energy metabolism rather than the protein utilization of the host animal.

The extent of microbial fermentation in the hindgut depends on the retention time of the digesta, which is influenced by different factors (animal species, dietary fibre, etc.). To provide information about microbiological modifications to the ingesta, germ-free or ileocaecal cannulated experimental animals can be used; however, antibiotics have the same effect if given orally.

The activity of the microflora in the hindgut affects apparent protein digestibility values by enhancing faecal endogenous nitrogen excretion. The amino acid pattern of the faeces is largely independent of the amino acid composition of the ingested protein as a consequence of the high ratio of endogenous faecal nitrogen (approximately 84–88%) to the total faecal nitrogen (Bergner and Bergner, 1983). The amount of endogenous faecal nitrogen corresponds to the sum of the real endogenous nitrogen (unabsorbed enzymes) and the microbial nitrogen.

Activity of the microflora in the hindgut has only limited influence on true protein digestibility and the biological value of unprocessed feedingstuffs determined in balance experiments (Bach-Knudsen et al., 1983). The reduction by Nebacitin of the gut microflora in rats fed diets high in crude fibre and low in protein resulted in a lower digestibility of the protein and the energy (Eggum et al., 1979).

Frequency of feeding and delayed supplementation of proteins or amino acids

The frequency of daily feeding has very limited influence on protein and energy utilization (Kirchgessner and Müller, 1980). Pigs have a greater tolerance in this respect than faster growing species (poultry, rats).

The free amino acids usually given as feed supplements may be absorbed at different rates compared to those bound in protein. This is, however, unlikely to cause any practical problems. Supplementary free amino acids are assumed to be wholly available. Delayed amino acid supplementation (1 or 2 hours after feeding) had only a slight effect on nitrogen balance (Yablonski and Rafalski, 1979).

When complementary proteins are fed at delayed times (one-, two- or three-day intervals), feed efficiency may be impaired because of different feed intakes resulting from differences in palatability (Mills and Canolty, 1984). Theoretically, all essential amino acids must be present simultaneously for optimum protein synthesis; however, a 2- to 4-hour lapse between the feeding of an incomplete mixture and complementary amino acids or supplementary proteins has only a very limited effect on protein utilization.

Animal species

Comparisons among rats, pigs and chickens fed the same protein source indicate that the digestibility data were the highest for pigs, followed by rats and the lowest values were obtained for chickens (Poppe and Meier, 1971). However, the values for energy as well as crude protein and amino acid di-

gestibility showed a high correlation between rats and pigs (Eggum, 1973). Absolute values for true protein digestibility (TD) and digestible energy (DE) obtained on rats and pigs did not differ significantly. Daily nitrogen retention in piglets in the weight range of 8–20 kg could be well predicted from utilizable protein (UP) values for rats (Eggum et al., 1987).

Chicks are capable of utilizing D-amino acid isomers of the branched-chain amino acids (leucine, isoleucine, valine) considerably better than rats. D-tryptophan is utilized efficiently by rats and pigs but inefficiently by chicks, poults, mice and humans. The D-forms of methionine, phenylalanine and tyrosine are utilized well by both rats and chicks. Avian species have a greater facility for utilizing amino-nitrogen groups, for synthesizing dispensable amino acids and for transforming alpha-keto analogues to their respective L-amino acids than do mammalian species (Baker, 1986).

Chicks were shown to be more sensitive to growth-depressing factors (trypsin inhibitors) than piglets.

Turkeys are more efficient in converting dietary protein to carcass protein than broilers (Summers et al., 1985).

Colostomized hens, 5-week-old chicks, adult mink, blue foxes and rainbow trout digested high-quality cod fillet with minor differences. The average amino acid digestibility in chickens was slightly below that of mink, foxes and rainbow trout. The feeding of low-quality meat-and-bone meal, however, resulted in considerable differences in amino acid digestibility between species as well as between different amino acids. The digestibility values of low-quality meta-and-bone meal obtained by mink and rainbow trout were significantly lower than those of other species (Skrede et al., 1980).

Mink may digest and utilize protein sources to a considerably lower extent than rats (Glem-Hansen and Eggum, 1974), however a close relationship was found between the two species for TD, BV and NPU ($r = 0.9$, 0.88 and 0.92, respectively).

A comparison of apparent protein digestibility of various protein sources in man and rat showed no significant differences (Rich et al., 1980).

Sex, age and health status

The higher growth potential of male animals shall be taken into account in protein quality assays based on weight gain. However, there appeared to be no significant difference between results obtained with males and females in nitrogen balance experiments (Eggum and Pedersen, 1983).

The influence of age on protein and energy utilization in adult rats was very limited (Fischer and Canolty, 1983). No differences have been found in the digestibility of organic matter in rabbits of 6–9 weeks and 4–12 months

of age; however, younger (8–9 weeks old) rabbits may digest protein significantly better (Fekete and Bokori, 1986). It is generally accepted that very young, post-weanling animals require time to develop appropriate digestive enzyme activities.

Specific pathogen free (SPF) rats grow more rapidly and give higher digestibility, BV and NPU values on the same protein source than conventional ones (Eggum, 1972). This is true also for SPF pigs. Germ-free chicks were shown to have a better feed efficiency than conventional animals (Furuse and Yokota, 1984).

Environmental temperature

At higher environmental temperatures (above 30 °C) feed intake may be limited. Excessive dietary amino acid levels may accentuate this effect (Waldroup, 1982). However, at temperatures below the thermal neutrality zone animals may burn proteins for energy and thus the efficiency of protein utilization for protein retention decreases.

Conclusions

The nutritional value of dietary protein depends primarily upon the amount and patterns of its essential amino acids. Therefore, the comprehensive evaluation of protein quality must start with the determination of nitrogen content (raw protein), amino acid composition, and digestibility. Finally, the capacity of proteins to fulfil nutritional needs is to be predicted. The potential available protein content of the feed will be utilized with different efficiencies during processes of protein metabolism, depending on the many factors influencing protein utilization, which are independent of the amino acid pattern of the dietary protein. If the protein quality of a feed is assessed by feeding experiments, these factors must be kept constant to obtain results comparable with those of other feeding experiments. A deeper understanding of the dietary factors influencing the efficiency of protein utilization helps to utilize feed protein resources more economically.

Acknowledgement

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BIOAVAILABILITY OF METHIONINE-HYDROXY-ANALOG FREE ACID AND S-METHYL-METHIONINE IN THE GROWING RAT

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The biological utilization of DL-methionine (MET), DL-methionine-hydroxy-analog (MHA) and DL-S-methyl-methionine-sulphonium-chloride (SMM) was tested in rat growth assay. Weight gain, feed efficiency, protein efficiency ratio (PER), net protein ratio (NPR) and net protein utilization (NPU) were applied as criteria. A test diet containing soybean meal as sole protein source was fortified with MET, MHA or SMM at 1.5 and 3.0 g/kg levels, respectively. All fortifications had a significant ($p < 0.05$) positive influence on weight gain, feed conversion and protein utilization. SMM showed the highest activity. MET and MHA were almost equally well utilized at 3.0 g/kg fortification level, however MHA proved slightly less efficient at 1.5 g/kg level than MET.

Keywords: Methionine, methionine-hydroxi-analog, S-methyl-methionine-sulphonium-chloride, protein utilization, feed conversion

The use of methionine as a feed supplement is relatively expensive; therefore, cheaper derivatives or substitutes with similar biochemical activity would be desirable. The L- and D-isomer forms of methionine are utilized almost equally well in the rat (Eggum and Pedersen, 1983); however, growth assays on a synthetic amino acid diet fed to mice showed that substituting D-methionine for the L-isomer resulted in a dose-dependent relative weight gain reaching approximately 76% when D-methionine was fed at a level equivalent to that optimal for the L-form (Friedman and Gumbman, 1984). The D-isomer of alpha-hydroxy-analog of methionine, however, proved to be more effective than the corresponding L-isomer (Friedman and Gumbman, 1989).

The compounds that might substitute or spare methionine are the naturally occurring sulphur amino acids (cystine, cysteine, homocysteine, etc.), the oxidized sulphur amino acids formed during processing of feeds (methionine-sulphoxide, methionine-sulphone, cysteic acid), and the analogs of methionine (S-methyl-methionine, methionine-hydroxy-analog, N-hydroxymethyl-methionine, etc.).

Cystine and methionine-sulphoxide had some sparing effect for methionine (Njaa and Aksnes, 1982), but methionine-sulphone proved unavailable in the rat (Miller and Samuel, 1970). Keto- and hydroxi-analogs of methionine

are utilized in the rat, chick, cow, sheep and rabbit, but the published efficiency of their utilization is conflicting (Schmidt and Sipőcz, 1977; Hartnell et al., 1984; Dibner et al., 1984; Baker, 1986; Fekete et al., 1990).

The study described in this report involved rat growth experiments to compare the effectiveness of methionine, methionine-hydroxy-analog and S-methyl-methionine in supplementing low-protein methionine-deficient diets using growth, feed conversion and protein utilization as criteria.

Materials and methods

The biological utilization of DL-methionine (MET, Merck), DL-methionine-hydroxy-analog (MHA, Monsanto), and DL-S-methyl-methionine-sulphonium-chloride (SMM, Reanal) was tested in a 10-day growth assay with weaning male rats (Wistar, SPF, LATI, Gödöllő, Hungary) using a methionine-deficient test diet.

Rats were housed in groups of six per wire cage. Feed and water were provided *ad libitum*. The temperature of the animal room was 28 °C and relative humidity was maintained between 60 and 80%. The animals were assigned so that all treatment groups had the same initial mean body weight.

The composition of the *test diet* was: cooking fat (lard) 14.25%, sunflower oil 0.75%, raw potato starch 10.0%, vitamin and mineral premix (Bábolna, Hungary) 5.0%, extracted soybean meal to give 10.0% crude protein in the test diet, and wheat starch to 100%. The test diet was fortified with MET, MHA and SMM respectively, at two levels (1.5 and 3 g/kg) to get the final diets. The composition of vitamin and mineral premix was adequate to meet the requirements of the rat (NRC, 1978).

A *protein-free diet* was used for determining the NPR and NPU indices. The composition of the protein-free diet corresponded to that of the test diet but wheat starch was used instead of soybean meal.

The criteria indicating the performance of the experimental rats fed on test diets were weight gain, feed efficiency, protein efficiency ratio (PER), net protein ratio (NPR), and net protein utilization (NPU). *Feed efficiency* was defined as weight gain (g) at the end of the feeding period per gram of feed consumed. *PER* was calculated as weight gain per weight of protein eaten. *NPR* is a modified index of PER and calculated as follows: weight gain of a test animal plus weight loss of the control animal fed a protein-free diet per gram of protein consumed by the test animal. *NPU* was calculated from the regression equation between NPR and NPU (NPU = 18.754 NPR; Hegedűs, 1986).

Results

The initial and final body weights of the rats and the intakes of feed, nitrogen and supplementary methionine source (MET, MHA, SMM) are summarized in Table 1. All values are averages of six rats. The final body weights marked with different letters deviate significantly ($p < 0.05$).

The final body weight of rats fed test diets fortified with 3.0 g/kg MET, MHA or SMM respectively was significantly higher than that obtained with an unfortified diet containing only soybean meal as a sole protein source. However, if a lower level of fortification (1.5 g/kg) was used, MHA did not support significant additional growth.

Feed conversion and protein utilization were characterized by weight gain, weight gain per weight of feed eaten (feed efficiency), weight gain per weight of crude protein eaten (PER), net protein ratio (NPR) and net protein utilization (NPU). These indices are shown in Table 2.

All of these parameters were improved if the test diet was fortified with 3.0 g/kg MET, MHA or SMM respectively. If the 1.5 g/kg fortification level of MHA was used, feed conversion and net protein utilization did not improve as compared to that obtained with an unfortified test diet.

In Table 2 the values of feed efficiency and NPU obtained using fortifications with MHA as well as SMM are expressed in per cent of those of MET. SMM exerted the highest while MHA showed the lowest activity in supple-

Table 1

Body weight and feed intake of rats fed on test diets fortified with methionine (MET), methionine-hydroxy-analog (MHA) and S-methyl-methionine-sulphonium-chloride SMM

Diets	Initial body weight (g/rat)	Final body weight (g/rat)	Feed intake (g/rat∗ 10 days)	N-intake (g/rat ∗ 10 days)	Intake of supplementary methionine source (rat ∗ 10 days)	
					mg	mmol
Control (protein free)	55.10±1.96	50.23±2.88	41.75	0.00	0.00	0.00
Test diet (A)	55.10±1.96	86.41±5.61 (a)	85.88	1.37	0.00	0.00
A+1.5 g MET/kg	55.10±1.61	97.91±11.1 (b)	106.62	1.71	159.93	1.07
A+1.5 g MHA/kg	55.10±1.71	92.66±4.93 (a)	101.22	1.62	151.83	1.01
A+1.5 g SMM/kg	55.10±1.69	101.08±9.00 (b)	106.27	1.70	159.41	0.80
A+3.0 g MET/kg	55.10±1.71	102.41±9.74 (b)	112.87	1.81	338.61	2.27
A+3.0 g MHA/kg	55.10±1.72	100.58±9.50 (b)	109.70	1.76	329.10	2.19
A+3.0 g SMM/kg	55.10±1.79	107.41±9.78 (b)	119.38	1.91	358.14	1.79

The test diet contained extracted soybean meal as a sole source of protein at 10% crude protein level
All values are averages of six rats
Values with different letters deviate significantly ($p < 0.05$)

Table 2

Feed conversion and protein utilization of rats fed on test diets fortified with methionine (MET), methionine-hydroxy-analog (MHA) and S-methyl-methionine-sulphonium-chloride (SMM)

Diets	Weight gain (g/10 days)	Feed efficiency (g/g) (a)		PER (g/g) (b)	NPR (g/g) (c)	NPU (%) (d)	
Control (protein free)	— 4.87	0.00		0.00	0.00	0.00	
Test diet (A)	31.31	0.36		3.65	4.23	79.23	
A+1.5 g MET/kg	42.81	0.40	(100%)	4.02	4.46	83.64	(100%)
A+1.5 g MHA/kg	37.56	0.37	(92.5)	3.71	4.19	78.57	(93.9)
A+1.5 g SMM/kg	45.98	0.43	(107.5)	4.33	4.79	89.73	(107.3)
A+3.0 g MET/kg	47.31	0.42	(100%)	4.18	4.61	86.49	(100%)
A+3.0 g MHA/kg	45.48	0.41	(97.6)	4.13	4.58	85.83	(99.2)
A+3.0 g SMM/kg	52.31	0.44	(104.8)	4.38	4.79	89.82	(103.9)

a = Weight gain per weight of feed eaten
b = Protein efficiency ratio
c = Net protein ratio
d = Net protein utilization

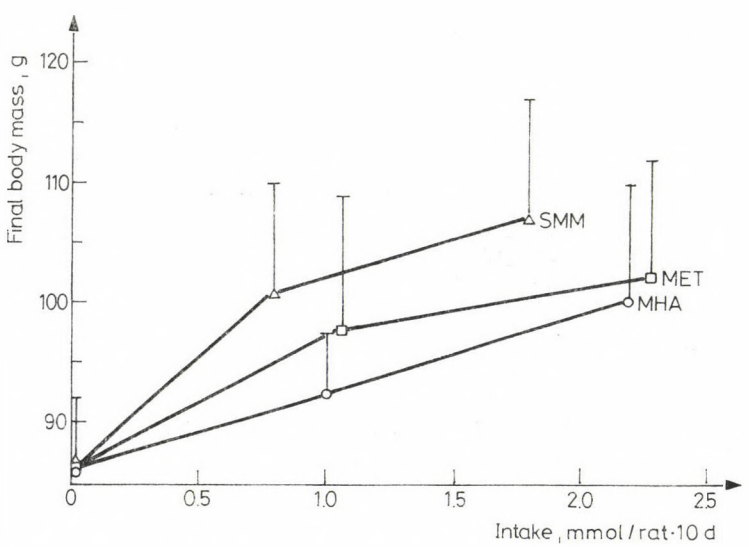

Fig. 1. Final body weight of rats plotted against supplementary methionine source eaten (SMM = S-methyl-methionine-sulphonium-chloride; MET = methionine; MHA = methionine-hydroxy-analog)

menting soybean protein. However, the differences in activity among MET, MHA and SMM at the 3.0 g/kg fortification level were insignificant.

Figure 1 shows the final body weight of the rats as a function of intake of supplementary MET, MHA and SMM respectively. The higher activity of SMM is obvious from the figure; however, differences in the effects of MET and MHA are negligible from the practical point of view.

Discussion

Methionine is the limiting amino acid in soybean protein, and its addition to the test diet containing soybean meal as sole protein source results in an improvement in net protein utilization.

The present study with rats shows that MET, MHA and SMM were utilized almost equally well when weight gain, feed efficiency and net protein utilization were applied as criteria. This demonstrates that the alpha-hydroxy-analog of MET can be dehydrogenated to an alpha-keto derivative and the latter can be transaminated to form L-MET at a sufficient rate to ensure nearly the same feed conversion and protein utilization as when the DL-MET is fed.

MHA is thought to be converted to L-MET in a way similar to that with D-MET. The technically producible racemate DL-MHA contains two isomers which must be converted biologically to L-MET, while DL-MET already consists of 50% of the L-form. On the other hand, MHA free acid consists of at least 20% dimer and trimer forms as a consequence of a condensation reaction between the molecules which first must be cleaved to biologically available monomer compounds. The lower efficiency of these transformations may account for the differences measured in the activity of DL-MET and DL-MHA.

In practical rations, in which the supplementation rate of DL-MET or DL-MHA accounts for only about 20–50% of the total sulphur-containing amino acids, it is difficult to monitor the difference in effectiveness between the two compounds. However, in supplementing MET-free diets with MHA the lower efficiency becomes more obvious.

The role of MET in the body is multiple. It readily donates its terminal methyl group for methylation of various compounds. The S-methyl derivative of MET has a higher activity in the rat than MET itself. This can be explained by the simultaneous supply of methyl group and MET for biochemical reactions in the body if SMM is used for fortifying the diets.

From the results of this experiment it can be concluded that the use of MET, MHA or SMM for fortification of a methionine-deficient diet is equally efficient if a 3.0 g/kg level is applied. At a lower (1.5 g/kg) level of fortification, however, MHA may exert lower activity than MET.

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BLOOD GLUTATHIONE PEROXIDASE ENZYME ACTIVITY AS AN INDEX OF SELENIUM RELEASE FROM PERMASEL® IN EWES

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Ten Merino ewes were given a single Permasel® pellet (containing 5% of elementary selenium) orally and examined for the release of selenium in the reticulum by determining glutathione enzyme activity of the whole blood haemolysate over a period of 12 months. As compared to the controls, the treated ewes exhibited a significant elevation in glutathione peroxidase activity for 8 months, indicating an acceptable persistence of the product tested.

Keywords: Blood haemolysate, ewe, glutathione peroxidase, Permasel®, selenium

One of the antioxidative defence mechanisms of the organism is the glutathione redox system. The selenium-dependent glutathione peroxidase enzyme, GSH-Px, is part of that mechanism (Little and O'Brien, 1968). In selenium deficiency the activity of GSH-Px declines. This may lead to peroxide intoxication, i.e. impaired protection against peroxidases which, in turn, may adversely affect the cell membranes and, thereby, most of the biochemical processes. This phenomenon is also called "oxidative stress" (Fridovich, 1984).

The lack of antioxidative effect of vitamin E, allowing deterioration of the cell membrane lipid layer, is basically caused by peroxides as well (Burton et al., 1983).

A selenium-deficient diet containing less than 5 ppm of this element (Nagy, 1978) may induce muscular dystrophy in lambs and piglets as well as exudative diathesis in chickens. These diseases can be prevented by administering selenium and/or vitamin E in the diet. As an alternative, Permasel® may be given to ruminants.

This long-acting selenium product was tested for efficacy and persistence in ewes by determining the glutathione peroxidase enzyme activity of blood haemolysate. The results are reported in this paper.

Materials and methods

Two random groups of 10 Merino ewes each were used. In the period of study lasting from June 1990 to June 1991, the sheep were kept on natural pasture in summer and on stubble fields in autumn. In winter the sheep were fed corn plant silage, grass hay and alfalfa hay. In addition, the ewes were also given concentrates in a daily portion of 0.4 kg per animal throughout the year except in summer.

The test group of ewes received Permasel®, a pelleted product of ICI containing 5% of elementary selenium and 0.5% of iron dust as carrier. One pellet was administered to each ewe with the aid of a special pair of pincers (via swallowing). Owing to its mass, the pellet gets into the reticulum and adheres there unremovably by movements of the forestomachs. As a result, a permanent selenium release takes place in the reticulum, supplying the organism with selenium independently of feeding.

The other 10 ewes served as the untreated control group.

Blood samples were taken from the ewes approximately at monthly intervals. The glutathione enzyme activity of whole blood 1 : 9 haemolysate was determined by the method of Matkovics et al. (1988). The enzyme activity was expressed as nkat (mmol glutathione oxidized per min at 25 °C) and corrected for 1 g protein content of haemolysate. The latter was determined by the Biuret method (Weichselbaum, 1946).

Means and coefficients of variation were calculated for the two groups and significance of the differences was tested by Fischer's F test (Sváb, 1973).

Results and discussion

The initial values of glutathione peroxidase activity for the treated and control ewes were practically identical (Fig. 1). One month after a single oral administration of Permasel® and subsequently the enzyme activity values obtained for the treated group were consistently higher than those of the controls.

The significant differences observed in glutathione peroxidase enzyme activity of whole blood haemolysate between the treated and control ewes (Table 1) indicate permanent release of selenium from Permasel® in the reticulum from August to April without any signs of selenosis.

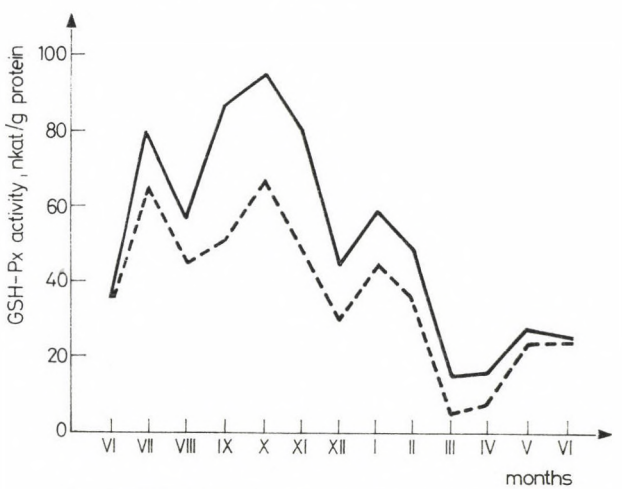
As the activity curves obtained for the two groups are parallel, the annual activity pattern of glutathione peroxidase in sheep can be characterized as follows. It is highest in autumn with a maximum in October, followed by an abrupt decline reaching minimum values in March–April; then from May it again tends to increase (Fig. 1).

Table 1

Evaluation of differences observed in mean glutathione peroxidase activity between selenium treated and control ewe groups

Month	Group	n	Differences in GSH-Px activity of whole blood haemolysate		
			Absolute (nkat/g protein)	Relative (%)	Significance level
VI	Se-C	20	2.12	6.03	NS
VII	Se-C	20	11.42	16.97	NS
VIII	Se-C	20	12.53	27.67	$P<0.05$
IX	Se-C	20	33.60	63.46	$P<0.01$
X	Se-C	20	30.71	46.11	$P<0.001$
XI	Se-C	20	31.50	64.28	$P<0.001$
XII	Se-C	20	14.07	43.41	$P<0.01$
I	Se-C	20	14.58	32.19	$P<0.05$
II	Se-C	20	16.55	44.79	$P<0.05$
III	Se-C	20	7.70	117.55	$P<0.001$
IV	Se-C	20	7.42	106.47	$P<0.001$
V	Se-C	20	1.90	6.97	NS
VI	Se-C	20	0.74	2.87	NS
Mean:		20	13.45	44.52	$P<0.01$

Se = selenium treated; C = control



Se-treated														
C.V.	18.1	21.2	14.8	27.3	7.5	15.0	26.9	18.7	28.0	36.5	21.9	12.3	6.5	
	11.7	10.4	24.2	19.7	24.1	10.8	10.8	22.0	18.8	32.7	28.4	19.9	22.1	
Control														

Fig. 1. Variations in glutathione peroxidase enzyme activity of ewes after a single oral administration of Permasel® (solid line: Se-treated ewes; broken line: control ewes)

This activity pattern may suggest that selenium administration to ewes shall be timed for late winter, particularly when alfalfa hay rich in vitamin E antagonists is given as staple diet. In such a case, the deficiency of selenium and of vitamin E (i.e. components of the antioxidative defence mechanisms capable of substituting for one another) may coincide, predisposing the animals to peroxide intoxication.

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IMPROVEMENT OF THE REPRODUCTIVE PERFORMANCE OF SOWS BY TREATMENT WITH A GnRH SUPERACTIVE ANALOGUE

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The effect of a Hungarian-made superactive analogue of GnRH (Ovurelin, D-Phe[6]-GnRH-EA, Reanal, Hungary) on the postpartal sexual function of sows was monitored. GnRH treatment was carried out on day 19 before weaning. The sows were inseminated at the first oestrus after weaning. GnRH treatment markedly increased litter size at weaning, substantially reduced (to 25 and 50%, respectively) the number of sows failing to come into oestrus after weaning, and increased the number of sows coming into oestrus within one week after weaning by 42,5% and 9%, respectively. These beneficial effects were particularly apparent on the far using closed management technology.

Keywords: Reproductive performance, sow, GnRH superactive analogue

Experience gained during the development of large-scale, industrialized pig production in closed management system indicates that the profitable operation of pig farms basically depends on the animals' reproductive performance. Optimal utilization of the herd's genetic capacity under the conditions of large-scale farming may require the use of biotechnological procedures including the administration of gonadotropin releasing hormone (GnRH) and its synthetic superactive analogues.

Sexual function is controlled by the hypothalamus-pituitary-gonad system also in sows. Certain neurons of the hypothalamus produce GnRH, which is a water-soluble oligopeptide consisting of 10 amino acids. This releasing hormone regulates the synthesis and release of gonadotropins (FSH, LH) produced in the hypophysis. In biological effect, the superactive analogues of GnRH are identical with natural GnRH; however, owing to their higher affinity to receptors of the pituitary gland they are much more effective than the former (Horváth et al., 1986; Nikolics et al., 1983).

In 1979, Buserelin (Receptal, buserelin acetate, Hoechst, Switzerland), one of the GnRH analogues was synthesized. According to the results of Mucsi and Buzás (1985), treatment of weaned sows with Receptal and vitamins A and E reduced the number of sows returning to oestrus by 10% and increased the conception rate by 7% within two oestrous cycles.

In an earlier paper (Szabó et al., 1991), we reported the effect of a Hungarian-made GnRH superactive analogue (Ovurelin, D-Phe^6-GnRH-EA, Reanal, Hungary) on the sexual function of sows after farrowing. Using the preparation 48 hours after weaning, we found that it did not improve the sows' reproductive performance; contrarily, it even prevented the manifestation of oestrus induced by weaning. At the same time, a regular cycle commenced in 96.8% of the animals.

In view of the above findings, we made further investigations which have opened up a new field of using the GnRH superactive analogue.

Materials and methods

First experiment. The first experiment was performed at a 1100-sow, closed, specialized pig farm of the Agricultural Combine of Mezőhegyes. Nineteen days before weaning, 69 sows were treated intramuscularly with GnRH superactive analogue (100 μg D-Phe^6-GnRH-EA, Ovurelin, Reanal, Hungary), while 103 sows that had farrowed simultaneously with the experimental animals and had been kept under the same management and feeding conditions, served as controls.

After weaning on day 30 $\pm$ 3 after farrowing, animals of both the experimental and the control group were transferred from the farrowing house of individual pen system to the sow house where they were kept in groups. Depending on the weather, the sows were allowed to spend 4–5 hours outdoors every day. After artificial insemination, the sows were kept in individual pens for 7–10 days, then were driven, in groups of 8, into a completely closed sow house having artificial ventilation and lighting. Sows possibly returning to oestrus were selected using teaser boars driven along the row of pens. Pregnant sows and those not returning to oestrus were transferred to the closed farrowing house having artificial ventilation and lighting one or two days before farrowing.

Second experiment. The second experiment was carried out on a 650-sow, specialized pig farm of the Agricultural Combine of Mezőhegyes. On that farm, weaning took place on day 30 $\pm$ 3 after farrowing. From the farrowing house, the sows were transferred to the sow house having an outdoor run, where oestrous animals were selected and inseminated artificially. Subsequently, four animals each were transferred to the inseminating house having large windows and excellent ventilation. Sows that did not return to oestrus by day 22 after insemination were grouped in 12-sow pens situated in the middle of the inseminating house, from where they were transferred to the sow house having an outdoor run at 55–60 days old. Three weeks before term the sows were transferred to the end of the farrowing house where they were

kept in 15-sow pens provided with an outdoor run and a resting place. After farrowing, the sows were exercised outdoors twice daily. In the predominant part of the reproductive cycle, the sows were either allowed free exercise outdoors or were staying in an excellently ventilated house having large windows.

On the latter farm, a total of 1045 sows were treated with GnRH superactive analogue on day 19 before weaning in 1987–1988. A total of 1195 weanings that took place in the same period of the years 1986–1987 served as control.

The onset of oestrus, as well as the times of return to oestrus, insemination and other reproductive events (e.g. abortion) were recorded regularly in all experiments.

Results

The results of the *first experiment*, presented in Table 1, indicate significant differences between animals of the two groups in the onset of postweaning oestrus. Sixty-six out of the 69 sows treated with the GnRH analogue 19 days before weaning (95.7%) came into oestrus within 7 days after weaning; in one sow the first oestrus occurred as late as 30 days after weaning. There were only two sows that failed to come into oestrus after weaning.

In the control group, 12 out of the 103 sows (11.7%) did not come into oestrus at all. Only 57 sows (55.3%) showed oestrous signs and were inseminated within 7 days after weaning. In 34 sows, the first oestrus took place only in the second week after weaning or later.

According to the data shown in Table 1.61 (88.4%) of the inseminated sows farrowed and produced a total of 601 piglets. Expressed for the original number of sows examined (n = 69), this corresponds to an average litter size of 8.7 piglets. In the control group, 68 of the oestrous and inseminated sows (66%) farrowed and produced a total of 679 piglets, which corresponds to an average litter size of 6.6 piglets for the 103 control sows examined.

In view of the observations made in the first trial, in the *second experiment* we studied, on a pig farm characterized by favourable reproductive-biological conditions, whether the proposed treatment results in sufficiently increased reproductive performance. The results are summarized in Table 2. It can be established that, even under sow-keeping conditions close to natural the treatment reduced the number of sows failing to come into oestrus after weaning to half (from 9% to 4.6%) and increased the number of sows coming into oestrus within one week after weaning from 52.4% to 61.2%. The average litter size per sow increased from 7.6 to 8.1.

Table 1

Reproductive results of sows treated with GnRH superactive analog 19 days before weaning (first experiment, closed management conditions)

Parameter studied	GnRH-treated group		Control group	
Number of animals tested	69		103	
Number of animals coming into oestrus after weaning	67	97.1%	91	88.3%
Animals showing oestrus on post-weaning				
day 2	2	2.9%	—	—
day 3	1	1.4%	11	10.7%
day 4	42	60.9%	13	12.6%
day 5	12	17.4%	15	14.5%
day 6	5	7.3%	8	7.8%
day 7	4	5.8%	10	9.7%
day 8	—	—	19	18.4%
day 9	—	—	4	3.9%
day 10	—	—	2	1.9%
day 11 or thereafter	1	1.4%	9	8.8%
Sows failing to come into oestrus in the period studied	2	2.9%	12	11.7%
Total number of sows that farrowed	61		68	
Total number of piglets born	601	88.4%	679	66.0%
Number of piglets per one sow included in the experiment	8.7		6.6	

Table 2

Reproductive results of sows treated with GnRH superactive analog 19 days before weaning (second experiment, management closer to natural conditions)

Parameter studied	GnRH-treated group		Control group	
Number of animals tested	1045		1195	
Animals showing oestrus on post-weaning				
day 3	4	0.4%	4	0.4%
day 4	134	12.8%	109	9.1%
day 5	300	28.7%	197	16.5%
day 6	133	12.7%	158	13.2%
day 7	69	6.6%	158	13.2%
day 8	56	5.4%	98	8.2%
day 9	28	2.7%	41	3.4%
day 10	16	1.5%	30	2.5%
day 11	13	1.2%	18	1.5%
Sows failing to come inte oestrus in the period studied	48	4.6%	107	9.0%
Total number of sows culled before weaning	123	11.8%	98	8.2%
Total number of piglets born	8460		9049	
Number of piglets per one sow included in the experiment	8.1		7.6	

Discussion

Gonadotropin releasing hormone (GnRH) and its analogues open up new possibilities in animal production.

Schlegel et al. (1984) conducted oestrus-induction and synchronization experiments on a total of 459 sows kept on an industrial-scale pig farm using GnRH vet. "Berlin-Chemie" analogue. The sows were weaned from their piglets after a 5-week nursing period, then were treated with 1000 IU of PMSG 24 h later. After further two days, the control group (n = 230 sows) was treated with 500 IU HCG, while the experimental group (n = 229) sows with 300 IU HCG and 300 μg GnRH analogue. All sows were inseminated "blind" twice, 24 and 48 h after the last treatment. Both treatment schemes resulted in a satisfactory oestrus rate (90.9 and 92.1%, respectively) and a good conception rate (84.9% and 88.1%, respectively). Bergfeld (1984) also performed that experiment on 172 weaned sows and 241 gilts and observed better results in the group treated with HCG + GnRH.

Raasch et al. (1984) applied the above treatment on a total of 1994 sows on 10 different pig farms and found that treatment with 300 IU HCG + + 300 μg GnRH proved to be better by 1.5–14.7%.

From postpartum day 31 $\pm$ 1 up to the 24th h after the onset of oestrus, Cox and Britt (1982) treated 6 sows with 1.5 μg GnRH intravenously every hour. GnRH administered in that way was found to stimulate oestrus during the lactation period and to induce LH and FSH production similar to that observed after weaning.

In this study, we tried out the GnRH superactive analogue on farms which markedly differed in terms of management technology. The animals were injected 19 days before weaning and were inseminated at the time of the first clinically apparent post-weaning oestrus. In this way we tried to make sure that GnRH-induced oestrus and oestrus brought about by weaning coincide.

According to the results, this procedure is especially suitable for increasing the reproductive performance of sows kept on closed, industrial-scale pig farms having artificial ventilation and lighting. In our second experiment, only 2 out of the 69 treated sows failed to come into oestrus after weaning. Of the 67 sows coming into oestrus, only one animal required more than one week (30 days) to show the signs of oestrus after weaning. Twelve out of the 103 control sows (11.7%) failed to come into oestrus, and only 55.3% of them showed oestrus within a week after weaning. This is why litter size in the GnRH analogue treated sow group exceeded that of the control group by more than 2 piglets on the average.

The second experiment was conducted on a pig farm using a management technology close to the natural conditions and markedly differing from

that used on the first pig farm. A total of 1045 sows were treated with GnRH superactive analogue as described above. As a result of treatment, the number of sows coming into oestrus within a week after weaning increased by 9%. The ratio of animals failing to come into oestrus decreased to half, and litter size expressed for the original number of sows tested increased by 0.5.

In summary, it can be stated that the treatment of sows with a GnRH superactive analogue 19 days before weaning increased the per sow litter size by markedly reducing the number of sows that fail to come into oestrus after weaning and by increasing the conception rate. These beneficial effects of the treatment were especially apparent on pig farms using completely closed management technology.

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SERUM PROGESTERONE, OESTRONE AND OESTRADIOL IN PREGNANT AND NON-PREGNANT RED DEER HINDS

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Changes in serum progesterone (P), oestrone (E_1) and oestradiol (E_2) concentrations were monitored in 34 female red deer *(Cervus elaphus)* shortly after the end of the breeding season and at mid-gestation. Pregnancy could be detected on the basis of serum P, but there were no significant differences between the pregnant and non-pregnant animals farmed animals in E_1 and E_2 concentrations. Twenty-five pregnant hinds captured in winter showed serum P levels similar to those found in farmed deer during the gestation period.

Keywords: Progesterone, oestrone, oestradiol, pregnancy, red deer

Cervides living in temperate latitudes, e.g. the Hungarian red deer *(Cervus elaphus)*, are characterized by seasonal polyoestrus (Guinness et al., 1971). The timing of the breeding season is determined genetically and is regulated by seasonal changes of the day-length (Lincoln, 1985). Increased ovarian activity results in a gradual increase of peripheral blood P concentration with a peak at about 14 days after the first ovulation (Adam et al., 1985). On the day before oestrus P concentration suddenly drops while E_2 concentration increases. At oestrus E_2 peaks four- to tenfold higher than that recorded for other polyestrous ungulates (Adam et al., 1985; Kelly et al., 1985). The lack of a prolonged period of low P concentration around oestrus has been observed (Kelly et al., 1985). Most hinds conceive during the first or second oestrus. Plasma P concentrations remain high during pregnancy until 1–4 weeks before parturition, when a rising E_2 concentration can be detected (Kelly et al., 1982; Adam et al., 1985).

The aim of this work was to collect data on serum hormone concentrations of pregnant and non-pregnant red deer. We were interested whether, in addition to serum P concentrations, serum E_1 and E_2 levels could also be used as an indicator of pregnancy.

Materials and methods

A total of 34 female farmed red deer (twelve yearlings, nine 2.5 years old animals and thirteen $\geq$ 3.5 years old deer) and 25 wild hinds (age range: 1.5–10.5 years) captured in winter (on 28 December) were used. The calves of the farmed hinds were weaned on 15 August. The matings with farmed stags of known fertility started on 1 September. Blood samples were taken from the farmed deer shortly after the end of the breeding season (on 8 November) and at mid-gestation (on 21 February) and from the captured wild hinds during the gestation period (on 21 February). The hinds were restrained in a crush during the sampling. Blood samples were drawn from the jugular vein, allowed to clot, centrifuged and the sera were stored at –18 °C until assayed.

Progesterone concentration was measured by the radioimmunological method of Poteczin et al. (1982). Serum E_1 and E_2 were assayed according to Poteczin et al. (1984).

Calving dates could be recorded only for the farmed hinds. Conception dates were calculated on the basis of a 234-day-long gestation (Adam et al., 1985).

The results were statistically evaluated by Student's *t* test.

Table 1

Serum progesterone (P) values (nmol/l) of farmed and captured Hungarian red deer hinds

Groups of hinds	n	Sampling data			Calving dates
		8 November $\bar{x}\pm$S.D.	Calculated for early post-conception days	21 February $\bar{x}\pm$S.D.	
PREGNANT					
FARMED					
Yearlings	7	4.2 2.4	21–43	18.4 8.2	18 May–9 June
range		(1.6–9.6)		(11.0–36.7)	
2.5-year-old	8	6.7 2.7	13–48	18.4 7.3	13 May–17 June
range		(2.2–11.2)		(10.2–32.6)	
3.5-year-old	12	4.8 1.1	16–52	13.4 5.3	8 May–15 June
range		(2.6–6.7)		(6.9–23.9)	
CAPTURED					4 May–3 June
1.5–10.5-year-old	25	—	—	14.9 4.6	
range				(7.2–24.3)	
NON-PREGNANT					
FARMED					
Yearlings	5	0.62 0.64		0.94 0.55	
range		(0.3–1.9)		(0.5–2.0)	
2.5-year-old	1	0.3		0.9	
4.5-year-old	1	0.9		0.7	

Table 2

Serum oestrone (E_1) and oestradiol (E_2) values (pmol/l) of Hungarian red deer hinds

Groups	n	Sampling data							
		8 November				11 February			
		E_1		E_2		E_1		E_2	
		$\bar{x}$	S.D.	$\bar{x}$	S.D.	$\bar{x}$	S.D.	$\bar{x}$	S.D.
Farmed hinds									
Non-pregnant	7	410	102.5	77	64.9	578	161.5	927	183.2
Range		(210–560)		(10–190)		(420–930)		(660–1240)	
Pregnant	27	439	224.0	106	61.1	596	184.6	1187	524.4
Range		(90–740)		(20–240)		(330–1040)		(660–2670)	
Captured hinds									
Pregnant	25	—		—		721	168.0	741	725.0
Range						(440–1020)		(150–3030)	

Results and discussion

As was expected on the basis of the high P concentration, 27 farmed deer calved between 8 May and 17 June. One of the 25 pregnant wild deer died, six of them aborted, and the remaining 18 calved between 4 May and 3 June. Seven farmed deer failed to conceive (5 yearlings, one 2.5-year-old and one 4.5-year-old hind). The conception dates of farmed deer fell between 17 September and 26 October while those of the captured deer between 13 September and 12 October. In farmed deer the first sampling (8 November) took place between the 13th and 52nd day after the calculated date of conception. With the exception of one yearling (P = 1.6 nmol/l on day 21 of pregnancy), pregnant farmed animals had serum P values exceeding 2.0 nmol/l (mean $\pm$ SD: 5.2 $\pm$ 2.3). The lowest P concentrations (2.2 and 2.6 nmol/l) were measured in animals being in the earliest phase of gestation, at day 13 and 16, respectively. On 21 February, pregnant farmed and captured hinds had similar P concentrations ($x \pm SD$: 16.9 $\pm$ 6.9 and 14.9 $\pm$ 4.6 nmol/l, respectively). Eight animals had serum P concentrations lower than 2.0 nmol/l at the first sampling. One of the six yearlings had a P concentration of 1.6 nmol/l; however, the second assay proved her to be pregnant (P = 13.4 nmol/l). Another animal had a P value of 1.9 nmol/l at the first testing but was found non-pregnant with a P concentration of 0.5 nmol/l at the second sampling. The P concentrations of the other six non-pregnant animals remained below 2.0 nmol/l (Table 1). Both pregnant and non-pregnant farmed animals had similar E_1 and E_2 values at the first sampling (Table 2). Oestrone concentrations were higher than E_2 concentrations in the hinds sampled

early. These data are similar to those recorded for white-tailed deer by Harder and Woolf (1976) and Plotka et al. (1977). At the second assay increased mean E_1 and E_2 levels were found both in pregnant and non-pregnant hinds. In that period E_2 levels were higher than E_1 concentrations. The E_2 values were significantly higher ($P < 0.001$) in pregnant and non-pregnant farmed deer, too. Oestrone values increased less markedly in pregnant ($P < 0.01$) and non-pregnant hinds ($P < 0.1$) between sampling dates. The results of this study suggest that E_1 and E_2 determination is not a suitable diagnostic tool to indicate pregnancy. It is interesting to note that in the captured deer serum E_1 concentration was higher ($P < 0.05$) and E_2 concentration lower ($P < 0.5$) than in the farmed animals. The results of the present study are consistent with those of Kelly et al. (1982). In red deer, serum P concentration appears to be a valuable tool for early pregnancy diagnosis owing to the significant ($P < 0.01$) differences demonstrable between pregnant and non-pregnant hinds. The assays can be performed shortly after the end of the main breeding season. In this study, serum P concentration was > 2.0 nmol/l in the pregnant and < 1.0 nmol/l in the non-pregnant hinds. In deer with P concentrations falling between 1.0 and 2.0 nmol/l (especially in yearlings) the determination of serum P concentration was less reliable as a diagnostic tool.

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GUIDELINES FOR THE ERADICATION OF INFECTIOUS BOVINE RHINOTRACHEITIS IN HUNGARY

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The prevalence of infectious bovine rhinotracheitis (IBR) in Europe is reported. Possible methods are proposed for eradication of infection as well as for the maintenance and control of infection-free status.

Keywords: Infectious bovine rhinotracheitis, eradication, guidelines.

Prevalence of infection

Infectious bovine rhinotracheitis (IBR) is widespread all over the world and occurs mostly as an enzootic disease (Ludwig and Gregersen, 1986). This applies to the majority of the Western European countries and to Hungary as well. In Belgium 62% of the cattle herds is seropositive and vaccination is practised widely to prevent the disease. In the United Kingdom, the 5% prevalence observed in the 1970's had increased to 15% and 45–50% in the breeding and producing herds, respectively, by 1988 (Ackermann et al., 1990). A survey conducted in 1978 in France revealed an average IBR prevalence of 10.8%. However, among Holstein-Friesian cattle the prevalence of infection was 27.5%; moreover, in Holstein-Friesian herds imported from Canada and the United States a seropositivity rate of 55.6% and 91%, respectively, was obtained (Perrin et al., 1981). In Germany 20–21% of the cattle herds was found to be infected (Bauer et al., 1980; Rothschuh, 1980; Forschner et al., 1988).

Norway, Sweden and Finland are almost free from IBR infection. In these countries IBR occurred only in a few foci of infection earlier, too. Denmark and Switzerland have launched a systematic eradication programme which is expected to result in infection-free status in 1991 or 1992 (Autrup and Bitsch, 1978; Ackermann et al., 1990).

In Hungary no nationwide statistical data are available on the prevalence of IBR infection. In exploratory surveys conducted in three Eastern counties between 1983 and 1988, a total of 160 herds were tested: of them, 28 (17.5%) proved serologically negative. In 105 herds (65.5%) a smaller part of the cows was serologically positive but the herd remained free from

clinical signs of the disease, while in 27 herds (16.9%) the majority (40–80%) of the cows were positive and IBR-induced abortions occurred, too (Tanyi et al., 1990).

Data on IBR infection of breeding bulls kept at Hungarian artificial insemination stations have been available since 1988. In a survey of two A. I. stations conducted in the first half of 1991, 49.3 and 48.9% of the bulls, respectively, proved to be positive (unpublished data).

Reasons for, and possible ways of, achieving infection-free status

Efforts aimed at achieving freedom from IBR are justified by the economic losses caused by the disease and the interests connected with the exportation of breeding animals. Only IBR-free breeding animals and semen or embryos obtained from such animals may be allowed for international trade. The majority of European countries stipulate freedom from IBR as a precondition of the importation of live cattle.

IBR can be eradicated by the selection of infected (seropositive) animals (Lucas, 1986; Corkish, 1988; Wyler et al., 1989; Ackermann et al., 1990), by the isolated rearing of calves (generation shift; Bradley, 1985; Tanyi et al., 1990), or by removing the infected herd and replacing it with a new, infection-free stock (herd replacement). Systematic, repeated vaccination of seropositive animals with an inactivated vaccine reduces the rate of virus shedding and, thus, contributes to the success of eradication by selection (Forschner et al., 1986; Meyer et al., 1985). All animals can be immunized with a vaccine containing a gene-deleted IBRV mutant strain and, using an appropriate blocking ELISA, the vaccinated cattle can be distinguished from those infected by virulent virus (Kit et al., 1990). Such a vaccine protects also the uninfected cattle during the period of selection and increases the safety of the selection procedure.

Methods proposed for achieving infection-free status

According to international experience and Hungarian findings obtained so far, the following methods can be recommended for achieving, maintaining and controlling IBR-free status of cattle herds in Hungary.

1. Selection

— In infected herds, all 6 months old or older animals must be subjected to a single serological testing using ELISA. Not only serum but also milk samples can be used for the test. Serologically positive individuals must be

separated from the negative animals; the latter must be kept isolated on a separate farm, barn or, in exceptional cases, within the same barn.

— Animals of the seronegative group must be subjected to serological testing repeated every 3 months and those reacting positively must be removed until all individuals of the herd give negative results by tests repeated every 2–3 months.

— Members of the seropositive group shall be vaccinated with inactivated vaccine twice at an interval of 3–4 weeks and subsequently every 4–6 months to reduce virus shedding to the lowest possible degree. No vaccine may be used in the seronegative group.

All animals of the herd to be eradicated may be immunized regularly with an IBRV vaccine containing a gene-deleted mutant (or some other marker) until infection-free status is reached or, if necessary, even further. Individuals infected by wild virus are selected (by an appropriate ELISA) and removed in the same way as described above.

— In principle, the above procedure is suitable for eradicating IBR infection from breeding bull stocks kept at A. I. stations and in other places. If the number of seropositive bulls is low at the station, they should be culled sooner or later even if they are kept isolated, or they should be transferred to stations where only infected bulls are kept. In this way the eradication of IBR from A. I. stations can be accelerated. Seropositive bulls shall be vaccinated in the same ways as seropositive cows.

— Serologically negative cows from IBR-free herds or herds currently under IBR eradication shall be mated only with infection-free sires, whether artificial insemination or natural service is used.

2. Eradication by generation shift

— All animals of serologically positive (infected) herds shall be vaccinated with an inactivated vaccine twice at an interval of 3–4 weeks and then repeatedly every 4–6 months to prevent the clinical signs and economic losses, and to reduce virus shedding.

— After weaning, the calves shall be reared isolated on a separate farm. From 6 months of age they shall be tested repeatedly by ELISA at an interval of 3–6 months until two consecutive tests give negative results for all animals. Seropositive growing cattle should be fattened and marketed as slaughter cattle.

— The growing heifer stock raised under infection-free conditions shall be reared further and bred in the same group, and shall be kept together until their calving. Growing heifers reared free from infection may be assigned only to IBR-free but at least seronegative cow herds.

— The calves of both seropositive and seronegative cows can be used for obtaining IBR-free growing stocks.

3. Eradication by herd replacement

All animals must be removed from the farm, the farm must be cleaned and disinfected thoroughly, subjected to repeated rodent control measures, and then stocked with a herd free from IBR.

Infection free status, its maintenance and control

A herd can be considered *free from infection* if two consecutive serological tests (ELISA) of serum or milk samples from all animals give negative results. Calves derived from seropositive cows shall be tested first at 3–6 months of age at the earliest. Namely, at a younger age calves having maternally derived antibodies are also seropositive; the levels of maternal antibodies, however, decline gradually and practically disappear by six months of age. At the same time, if infections occurs before the animal reaches the age of six months, the antibody titres will increase and the animal remains seropositive for a long time.

Infection-free status can be maintained by ensuring closed managemental conditions as far as possible. Contact and mixing of the herd with other herds or animals must be avoided, artificial insemination, infection-free bulls or semen obtained from infection-free sires must be used. Only infection-free sires shall be used at the A. I. stations. As the eradication progresses, only infection-free animals shall participate in the trade of breeding animals, first only in the infection-free areas and then on a nationwide scale. Only animals derived from infection-free herds shall be introduced to the IBR-free herds: by serological tests using serum or milk samples these animals shall give negative result at the time of purchase and then again after a one-month quarantine.

For *controlling the infection-free status*, all breeding sires and at least 10% of the cows in each herd shall be tested for IBR by ELISA every 6 months. Milk samples can also be used for these tests. If a seropositive animal or animals are detected in the herd, all individuals must be subjected to serological testing. If the prevalence of infection is 1% or less, the infection-free status of the herd can be maintained by removing the seropositive animals. If the number of seropositive animals is higher than that, the procedure described under eradication by selection shall be adopted.

IBR-free herds must be free also from tuberculosis, brucellosis and leucosis. During the eradication of IBR, it is advisable to pay attention to paratuberculosis and bovine viral diarrhoea as well.

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PARATUBERCULOSIS VACCINE IN A LARGE DAIRY HERD

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On a 500-cow dairy farm a total of 866 young calves less than one month old were vaccinated with a heat-killed oil-adjuvated bacterin against *Mycobacterium paratuberculosis* over a period of five years. The vaccinated calves were tested by faecal microscopy, bacteriology and serology on the day of vaccination, at the age of 3, 6, 9 and 12 months, at breeding age, and on the day of calving. A total of 721 bull calves and 379 female calves served as unvaccinated controls in two groups. The results were evaluated by trend analyses.

Vaccination greatly reduced the faecal shedding of mycobacteria as demonstrated by the annual faecal microscopic examinations. During the last 6 months of the experiment only 9 of 612 samples were found positive by microscopy and by bacterial culture. The number of seropositive animals and the antibody titres demonstrated by the complement fixation test (CFT) and agar gel immunodiffusion (AGID) increased during the first three years. Later on, both the number of seropositive animals and CFT titres decreased.

Keywords: Cattle, paratuberculosis, vaccine

Paratuberculosis is an infectious disease of ruminants causing significant economic losses worldwide (Amstutz, 1984; Körmendy et al., 1989*a*). Although its public health significance is questionable, a high proportion of animal health professionals and farmers seem to have demonstrable antibodies against *Mycobacterium paratuberculosis* and in some situations a disproportionately high incidence of Crohn's disease was found in such persons (Chiodini, 1988).

Vaccination is one of the means of combatting the disease. In the last 60 years, live and killed vaccines were prepared in several countries to minimize economic losses caused by paratuberculosis (Vallée and Rinjard, 1926; Stableforth and Galloway, 1959). Limited information is available, however, about the effect of vaccination on faecal shedding of *M. paratuberculosis* and on the results of immunodiagnostic tests.

So far, live (Berg-Jorgensen, 1984; Halgaard, 1984) and killed bacteria, as well as disrupted *M. paratuberculosis* cells (Larsen et al., 1969; Larsen et al., 1978; Larsen and Merkal, 1979) have been used in vaccination experiments. The advantages (safety, marketing, storage, bacterial shedding) and disadvantages (moderate protective effect) of killed vaccines are well known (Larsen et al., 1978; Larsen and Merkal, 1979; Huitema, 1967).

In order to learn more about the effect of vaccination on faecal shedding of bacteria and on the results of serodiagnostic tests, vaccination with a killed vaccine was performed in a dairy herd over a period of five years and the results of faecal and serological diagnostic tests were monitored.

Materials and methods

The vaccine was a heat-killed (121 °C, 30 min), oil-adjuvated bacterin prepared from *M. paratuberculosis* 5889 Bergey strain. In the first year of the experiment, the dose of the vaccine (containing 25 mg of killed bacteria per ml) was 4 ml administered intramuscularly. Subsequently, the dose was reduced to 1 ml i.m.

The experiments were conducted on a dairy farm having 500 Holstein-Friesian cows kept on deep litter in loose housing system. The pregnant animals calved in small groups. On the second day after calving, newborn calves were transferred to individual cages where they were kept up to weaning age. Weaned calves were grouped by sex.

Experimental group. Vaccination was carried out between 1 June 1985 and 30 June 1990. A total of 866 newborn and young (< 1 month old) female calves were vaccinated.

Blood and faecal samples were collected from the animals on the day of vaccination and subsequently at 3, 6, 9 and 12 months of age, at breeding age and on the day of calving.

Control group 1. Since bull calves were slaughtered at the age of 6–8 months, a full-term unvaccinated control group could not be created. These calves were examined using the same system as were female calves up to that age. A total of 721 bull calves were examined.

Control group 2. This group comprised 379 unvaccinated newborn female calves of another herd infected by *M. paratuberculosis*. These animals were kept under a similar housing system as the experimental group and were examined in the same way as the experimental group.

Allergic tests. All the cows and part of the heifers were tested simultaneously using bovine and avian allergens in intradermal tests carried out yearly as described by Körmendy et al. (1990).

Serological tests. Complement fixation (CFT) and agar gel immunodiffusion (AGID) tests were carried out (Körmendy et al., 1984) using positive and negative reference control sera as described by Körmendy et al. (1989*b*).

Microscopy and culture of faecal samples. For microscopic examination and bacterial culture faecal samples of approx. 10 gram each were collected and examined according to the guidelines of the Veterinary Service Laboratory, APHIS, USDA (1974). Bacteria were cultured on Herold's medium improved

with sodium pyruvate and mycobactin (Berg-Jorgensen, 1982). In 1990, the positive results of faecal microscopy were confirmed by bacterial culture.

Mathematical analyses. Trend analyses were carried out using Harvard programme, and the results were illustrated in graphs.

Results

Before the vaccinations the prevalence of *M. paratuberculosis* infection in the herd of 500 cows was as follows: 155 cows (31%) were found positive by faecal microscopy, 60 cows (12%) were positive by CFT, 54 cows (10.9%) were positive by AGID, and 15 cows (3%) showed positive or doubtful reaction to bovine allergen by the intradermal test.

The results of serological and faecal tests of animals of different age were recorded continuously after the vaccinations. These results are summarized in Table 1 and their trends are illustrated in Figs 1 and 2.

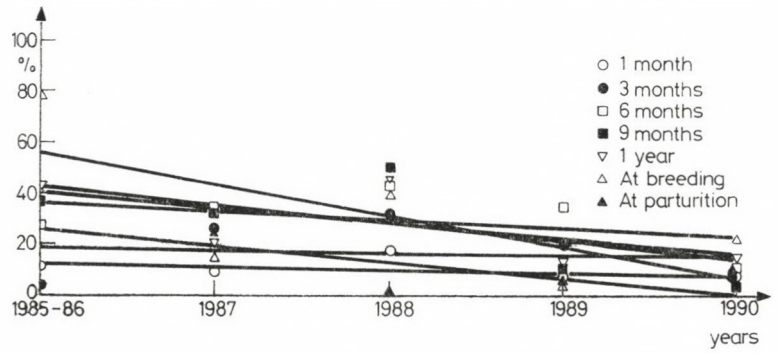

Fig. 1. Seropositivity rate (%) in the group of vaccinated female cattle (not tested at parturition in 1985–1986)

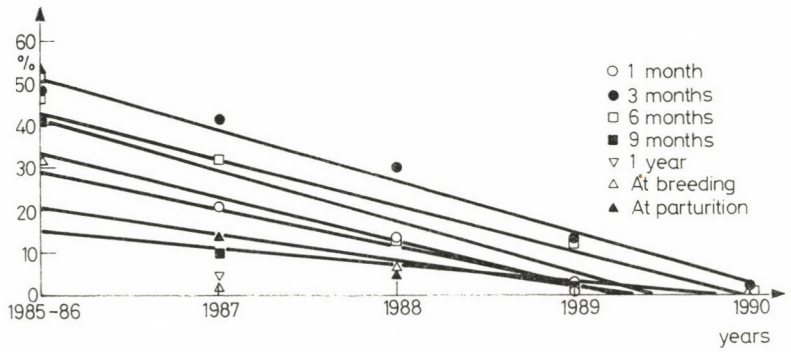

Fig. 2. Faecal positivity rate (%) in the group of vaccinated female cattle (not tested at parturition in 1985–1986)

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Table I

Ratio of seropositive animals and animals positive by faecal microscopy and culture among vaccinated female cattle

Age of female cattle	1985—1986			1987			1988			1989			1990		
	n	S+%	F+%	n	S+%	F+%	n	S+%	F+%	n	S+%	F+%	n	S+%	FC+%
1 month (before vaccination)	279	11	51	163	9	21	164	17	12	158	6	—	102	8	2
3 months	214	4	48	147	26	41	145	31	30	144	19	13	93	3	2
6 months	131	27	46	134	32	32	157	43	13	137	34	12	85	11	2
9 months	91	37	40	123	31	9	152	49	5	136	11	5	75	12	—
12 months	30	43	53	111	20	3	138	44	4	131	13	2	103	14	2
At breeding age	19	78	31	95	13	2	143	39	6	97	4	—	103	22	1
At calving				15	26	13	116	17	4	146	5	3	51	4	—
Total:	764	18	48	788	22	20	1015	35	11	949	13	5	612	11	1

n = number of animals tested; F+ = positive by faecal microscopy; S+ = seropositive by CFT or AGID; FC+ = positive by faecal microscopy and culture

The total numbers of CFT, AGID, faecal microscopic examinations and bacterial culture are shown in Table 2, together with their results expressed in per cent.

Regular intradermal tuberculin testing was performed between 1986 and 1990 once a year using bovine and avian allergens. The number of positive and doubtful allergic reactions to bovine and avian tuberculin increased from 5.8 to 37.0% and from 8.9 to 41.9%, respectively, by the 4th year (Table 3).

Regardless of the animals age, bovine tuberculin had a share of 43.4% (426) while avian tuberculin 56.6% (555 animals) of all reactors (2824 animals). These results are shown in Table 3 and Figs 3 and 4.

Table 2

Summarized results of serologic tests and faecal microscopic examinations of the vaccinated heifers (with all age groups represented)

Year	Number animals	Seropositivity, %	Faeces +,%
1985—1986	764	17.9	48.0
1987	788	22.4	20.0
1988	1015	35.1	11.1
1989	949	13.4	4.5
1990	612	10.9	1.4*

*positive by faecal microscopy and culturing

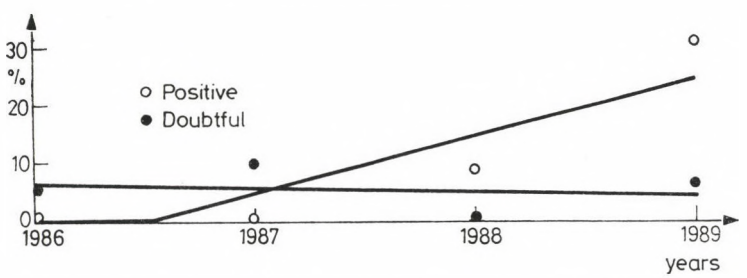

Fig. 3. Allergic reactions to bovine tuberculin in the group of vaccinated heifers and cows

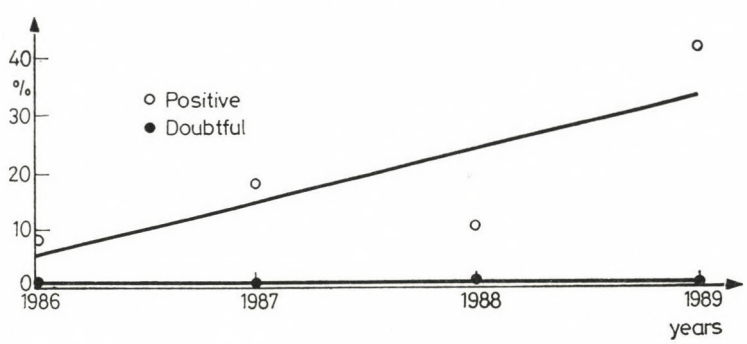

Fig 4. Allergic reactions to avian tuberculin in the group of vaccinated heifers and cows

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Table 3

Results of skin tests of vaccinated female cattle with bovine and avian allergens

	1986			1987			1988			1989			1990
	n	+	±	n	+	±	n	+	±	n	+	±	
With bovine tuberculin													
Cows	454	2	9	437	—	9	407	12	—	449	142	36	NT
Heifers, 1—2 years old	245	1	4	157	—	24	116	14	2	150	42	6	
Heifers, <1 year old	135	1	32	81	—	33	105	30	—	83	27	—	
Total:	834	4	45	675	—	66	628	56	2	682	211	42	
%		5.8			9.7			9.2			37.0		
With avian tuberculin													
Cows	454	6	3	437	15	—	407	12	6	449	184	3	
Heifers, 1—2 years old	245	6	1	157	41	—	116	53	1	150	72	—	
Heifers, <1 year old	135	55	4	81	66	—	105	NT	NT	83	27	—	
Total:	834	67	8	675	122	—	628	65	7	682	283	3	
%		8.9			18.0			11.4			41.9		

NT = not tested; + = positive; ± = doubtful; % = positive and doubtful

Results broken down according to the month of the year showed that twice as many faecal samples were found positive in calves born in March and April as in those born in January, February and in the summer months (results not shown in detail).

The number of positive faecal samples (microscopy) and the level of antibodies were found to increase in the first three years. The serum antibody titre reached 1 : 160.

Local reactions were frequently observed when the 4 ml vaccine dose was used. However, no local reaction was observed after using 1 ml vaccine in the last 4 years of the experiment.

Infection rate decreased continuously in the unvaccinated group of bulls during the experiment. At the end of the experiment only two bulls were positive by faecal culture (Table 4; Figs 5 and 6).

In almost every year of the experiment, the results of blood and faecal tests of the unvaccinated heifers were found unchanged as compared to the beginning of the experiment (Table 5; Figs 7 and 8). In this group, the highest

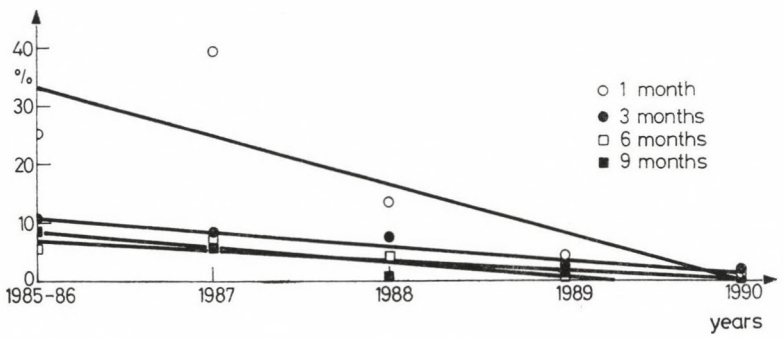

Fig. 5. Faecal positivity rate (%) in the group of unvaccinated bulls

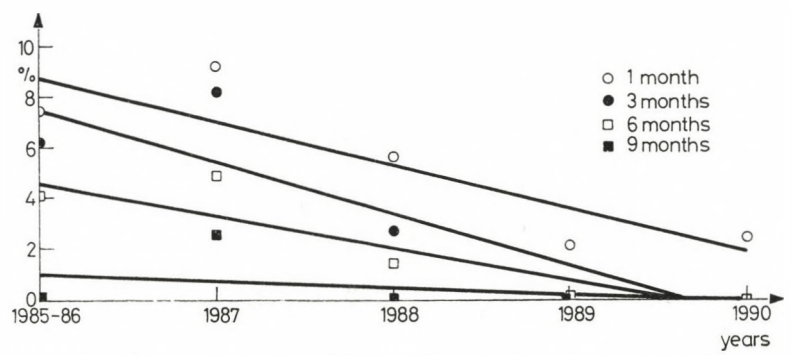

Fig. 6. Seropositivity rate (%) in the group of unvaccinated bulls

Table 4

Ratio of seropositive animals and animals positive by faecal microscopy among unvaccinated control bulls

Age, months	1985–1986			1987			1988			1989			1990		
	n	CFT+ %	F+ %	n	CFT+ %	F+ %	n	CFT+ %	F+ %	n	CFT+ %	F+ %	n	CFT+ %	F+ %
1	243	7.4	25.5	130	9.2	39.2	125	5.6	13.6	140	2.1	4.2	83	2.4	1.2
3	191	6.2	10.9	97	8.2	7.8	110	2.7	7.2	90	—	2.2	69	—	1.4
6	147	4.0	5.4	83	4.8	7.2	70	1.4	4.2	43	—	—	65	—	—
9	126	—	8.7	80	2.5	6.2	30	—	—	—	—	—	—	—	—
Total:	707	5.0	14.4	390	6.6	20.2	335	3.2	8.3	273	1.0	2.9	217	0.9	0.9

CFT+ = complement fixation test positive; F+ = positive by faecal microscopy; FC+ = positive by faecal microscopy and culture

Table 5

Ratio of seropositive animals and animals positive by faecal microscopy and culture among unvaccinated control female cattle

Age of female cattle	1985–1986			1987			1988			1989			1990		
	n	S+ %	F+ %	n	S+ %	F+ %	n	S+ %	F+ %	n	S+ %	F+ %	n	S%	FC+ %
1 month	82	6.0	41.4	63	6.3	46.0	89	10.1	48.3	101	7.9	45.5	44	11.3	43.1
6 months	59	6.7	20.3	55	9.0	21.8	80	7.5	23.7	87	6.8	21.8	36	11.1	19.4
12 months	47	9.4	23.4	50	12.0	24.0	71	9.8	22.5	69	10.1	20.2	30	0.0	20.0
At calving	—	—	—	41	7.3	14.6	60	8.3	18.3	58	13.7	20.6	22	9.0	18.0
Total:	188	7.4	30.3	209	8.6	28.6	300	9.0	29.6	315	9.2	28.8	132	10.6	27.2

n = number of animals tested; S+ = seropositive by CFT or AGID; F+ = positive by faecal microscopy; FC+ = positive by faecal microscopy and culture

Table 6

Results of skin tests of unvaccinated heifers

	1986			1987			1988			1989		
	n	+	±	n	+	±	n	+	±	n	+	±
With bovine tuberculin												
Heifers, 1—2 years old	—	—	—	43	—	2	61	—	3	51	1	2
Heifers, <1 year old	62	1	2	51	—	2	33	—	2	27	—	1
Total:	62	1	2	94	—	4	94	—	5	78	1	3
%		1.6	3.3		—	4.2		—	5.3		1.2	3.8
With avian tuberculin												
Heifers, 1—2 years old	—	—	—	43	1	3	61	1	4	51	1	3
Heifers, <1 year old	62	2	4	51	—	4	33	1	2	27	—	1
Total:	62	2	4	94	1	7	94	2	6	78	1	4
%		3.2	3.2		1.0	7.4		2.1	6.3		1.2	5.1

+ = positive; ± = doubtful; % = positive and doubtful

infection rate was found by faecal culture and microscopy of samples from the one-month-old animals.

The overall positivity of faecal samples varied between 27.2 and 30.3% in different years of the experiment.

Allergic tests proved to have low sensitivity in detecting the infection (Table 6; Figs 9 and 10).

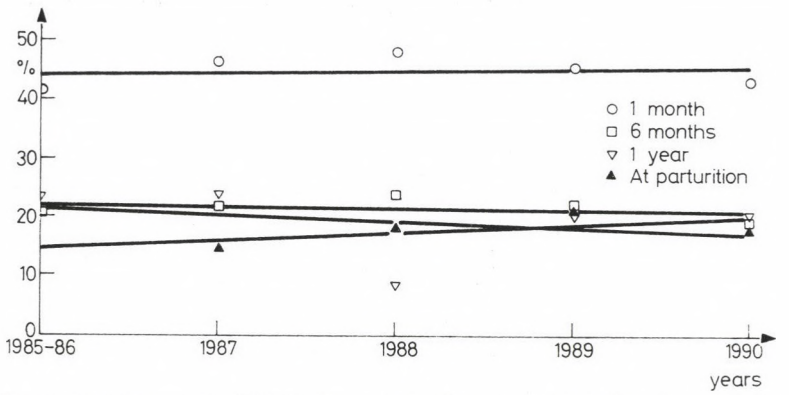

Fig. 7. Faecal positivity rate (%) in the group of unvaccinated female cattle (not tested at parturition in 1985–1986)

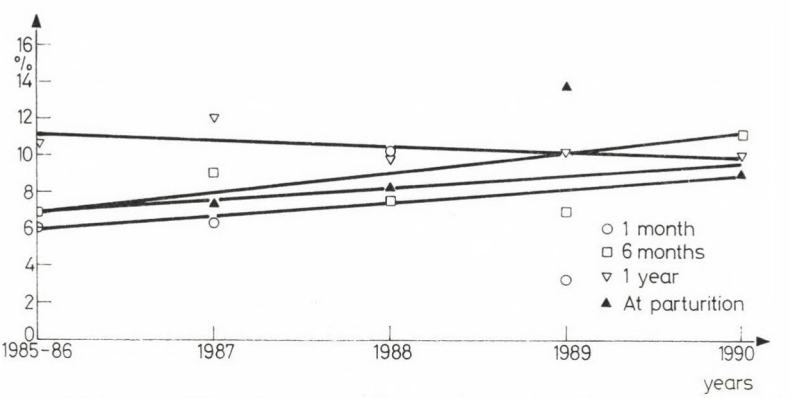

Fig. 8. Seropositivity rate (%) in the group of unvaccinated female cattle (not tested at parturition in 1985–1986)

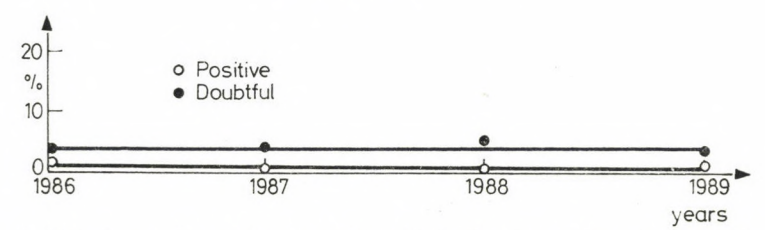

Fig. 9. Allergic reactions to bovine tuberculin in the group of unvaccinated heifers

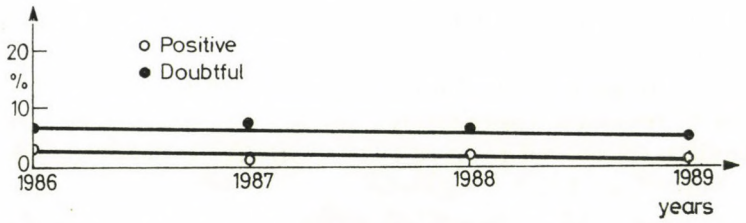

Fig. 10. Allergic reactions to avian tuberculin in the group of unvaccinated heifers

Discussion

Although isolated housing of infected animals is very effective in reducing losses due to paratuberculosis (Thoen and Moore, 1988; Amstutz, 1984; Chiodini et al., 1984), in most cases it is not possible and financially not feasible under normal farming conditions. Changes of management are also known to influence the outcome of infection (Amstutz, 1984; Larsen and Merkal, 1968). Therefore, in order to get a more realistic picture about the value of the vaccine, the management system of cattle was not changed in any of the herds investigated in this study.

Over a period of five years, calves vaccinated with a killed *M. paratuberculosis* bacterin were monitored from one month of age to their first, second and third calving for their immunobiologic responses in different diagnostic tests. CFT and AGID were found to detect antibodies and the number of seropositive samples increased from 17.9 to 35.1% during the first three years of the experiment. Serologic tests performed at one month of age indicated that the antibodies had been passed from cow to calf. The possibility that maternal antibodies could provide a certain level of protection was proposed by Rankin in 1957 (cit. Stableforth and Galloway, 1959); however, later on it was less well recognized. Our results support Rankin's findings and emphasize the importance of immunotherapy which has recently been taken into consideration again (Stanford et al., 1990).

The prevalence of paratuberculosis as assessed by faecal microscopic examinations decreased from 48% to 1.4% in the vaccinated group. In the unvaccinated control group, however, *M. paratuberculosis* bacteria were found in 27.2–30.3% of faecal samples taken from heifers. Vaccinated pregnant heifers began to calve 2.5 years after vaccinations had been started. Consequently, decreasing numbers of calves were born to the unvaccinated cows up to the end of the experiment.

This situation offers a new outlook on the value of the vaccine. However, our data about a possible colostral protection could not be evaluated.

Although the number of infected animals (which were kept on a different farm) remained unchanged among the unvaccinated control heifers throughout

the experiment, the tests of unvaccinated bulls gave more favourable results in the last two years of the experiment with respect to the first four years. This was probably due to the decreasing number of bacteria present in the environment of this latter group as a result of reduced bacterial shedding by vaccinated heifer calves. The heifers and bulls were kept unisolated until 8–9 months of age. Although the vaccine essentially reduced the shedding of bacteria in the faeces, it did not eliminate the infection, consistently with earlier reports (Stuart, 1965; Doyle, 1964; Larsen et al., 1978). In this case, this was confirmed by faecal culture in the last year of the experiment.

Although the protective effect of vaccines was known (Stuart, 1965; Larsen et al., 1978; Halgaard, 1984), the influence of the cows' age on the effectiveness of vaccination and other details have not been studied by diagnostic methods on large farms in sufficient detail.

As after the vaccination of calves or heifers it was impossible to distinguish between bovine and other type of infection by allergic tests, we proposed a strict check for tuberculosis at abattoirs and the examination of every suspicious case by histological and bacteriological methods. No signs of bovine tuberculosis were found.

Further development of *M. paratuberculosis* vaccines would require more information on different virulence factors relative to vaccines which are still missing at present. The role of cytokines, particularly Tumor Necrosis Factor (Rook et al., 1990) and phospholipases (Wheeler, 1991), should also be clarified in more detail in order to improve methods of immunological protection against paratuberculosis.

Although we could not give satisfactory explanation for the increase of antibody levels, the vaccine probably played a role in it.

On the basis of results obtained in this experiment, we propose vaccination as a means of reducing the infection rate in herds where the management system cannot be changed (Fig. 2).

The effect of this vaccine on the economy of production will be reported in a forthcoming paper.

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OCHRATOXIGENIC MOULDS AND OCHRATOXIN A IN FORAGES AND GRAIN FEEDS

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The contamination of forages and grain feeds with ochratoxigenic moulds and ochratoxin A was examined. The investigations were carried out over a period of three years in all seasons.

Feeds were found to be contaminated with moulds at a high level throughout the three research years. The highest percentage (95 to 100) of contaminated feed samples was noticed during the second year. Total viable counts of moulds established in 1 g of feed samples ranged from 0.5 to 7.8 $\times$ 10^6.

Penicillium spp. were dominant in mycopopulations isolated from feeds. Ochratoxin-A producing moulds were present permanently. In the summer period of the second research year as much as 94% of the feed samples were contaminated by ochratoxigenic *Penicillium* species. *P. verrucosum* var. *cyclopium P. verrucosum* var. *verrucosum*, *P. commune* and *P. chrysogenum*, i.e. ochratoxin-producing moulds, were the most prevalent *Penicillium* species throughout the three-year investigation.

Ochratoxin A was found in various feeds in all seasons, except in summer of the first research year. Concentrations of the toxin varied from traces to 400 μg/kg. It occurred consistently in the same types of feeds (hay, dried alfalfa, fresh alfalfa, concentrate, pelleted sugar beet pulp, corn silage),

Keywords: Ochratoxin A, ochratoxigenic moulds, forages, grain feeds, contamination

Ochratoxin A is a toxic metabolite of several *Aspergillus* and *Penicillium* species that are often present in various foods and feeds. Vengušt et al. (1983) reported that out of 305 hygienically unsuitable feedstuff samples, originated from 53 regions of Yugoslavia in 1979 through 1982, 65% were mouldy and as much as 27% were contaminated with ochratoxin A. *Aspergillus* isolates were found to be most widely distributed in the majority of feedstuff samples collected from different factories in Algeria in 1987—1988 (Atalla and El-Din, 1990). About 25% of all fungal isolates belonged to the *Aspergillus ochraceus* group and most of them were able to produce ochratoxin.

Ochratoxin A, a mycotoxin mainly occurring in grains, has been shown to be a potent nephrotoxin and hepatotoxin in all species of animals tested (Krogh et al., 1977; Pavlović et al., 1979; Petkova-Bocharova and Castegnaro, 1985; Petkova-Bocharova et al., 1988). There is also a hypothesis that ochratoxin A is associated with Balkan endemic nephropathy.

The purpose of this three-year study was to investigate the occurrence of ochratoxigenic moulds in forages and grain feeds, their prevalence, and the possible presence of ochratoxin A.

Materials and methods

The test forages and grain feeds originated from four large state farms in Vojvodina. The investigations were carried out over a period of three years in all seasons.

The determination of total viable counts of moulds per g of feed and their isolation were performed using Koch's standard method. Sabouraud dextrose agar with streptomycin (0.01–0.02%) was used as an isolation medium. Incubation was carried out at 25 ochratoxin A for 5 to 7 days. Identification of mould species was performed according to Raper and Thom (1949), Raper and Fennell (1965), Samson et al. (1976) and Samson and van Reenen-Hoekstra (1988).

Qualitative and quantitative determination of ochratoxin A was carried out according to the method described by Balzer et al. (1978). Pure ochratoxin A from *Aspergillus ochraceus* was supplied by Fluka Biochemika 7411 (Switzerland).

Results and discussion

The results given in Figs 1, 2 and 3 show that the contamination level of feeds by moulds was relatively high throughout the three-year period. The highest percentage of contaminated feed samples was found during the second research year (summer and spring: 100%, fall and winter: 95%; Fig. 2). The contamination level found in the first and third research year was somewhat lower but still high (first year: 82 to 95%; third year: 83 to 96%). Total viable counts of moulds ranged from 0.5 to 7.8×10^6 per g of feed.

Ochratoxin A-producing moulds were consistently isolated from feeds. It is necessary to point out that in the mycopopulations isolated *Penicillium* species such as *P. verrucosum* var. *cyclopium*, *P. verrucosum* var. *verrucosum*, *P. commune* and *P. chrysogenum* were dominant throughout the three-year study. On the other hand, moulds of the *Aspergillus ochraceus* group occurred very rarely. These fungi did not occur in the first research year (Fig. 1), but in the second year 9 to 12% of the feed samples were found to be contaminated with them. Besides *A. ochraceus*, *A. sulphureus* (summer) and *A. sclerotiorum* (winter) were present.

Ochratoxin A-producing *Penicillium* species had a significant share in mycopopulations isolated from the feeds. This conclusion applies especially to

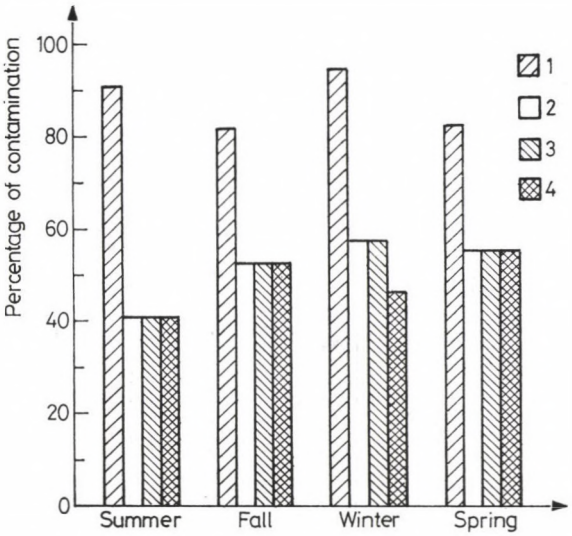

Fig. 1. Share of ochratoxin A-producing moulds in mycopopulations isolated from dairy cattle feeds during the first year of study. 1: moulds; 2: ochratoxin-producing moulds; 3: *Penicillium* spp.; 4: *P. verrucosum* var. *cyclopium*

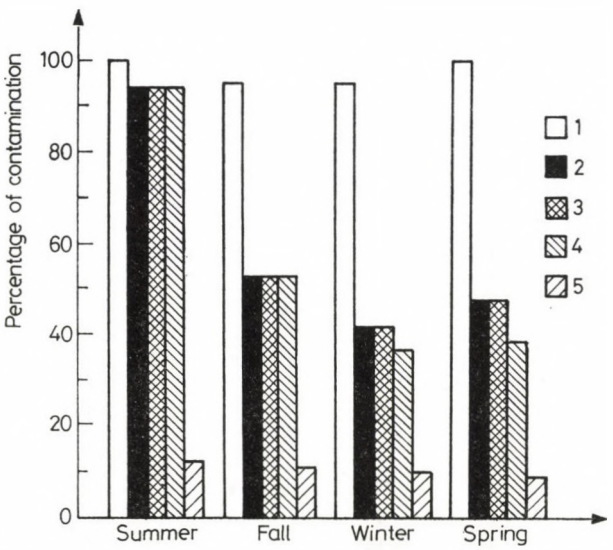

Fig. 2. Share of ochratoxin A-producing moulds in mycopopulations isolated from dairy cattle feeds during the second year of study. 1: moulds; 2: ochratoxin-producing moulds; 3: *Penicillium* spp.; 4: *P. verrucosum* var. *cyclopium;* 5: *Aspergillus ochraceus* group

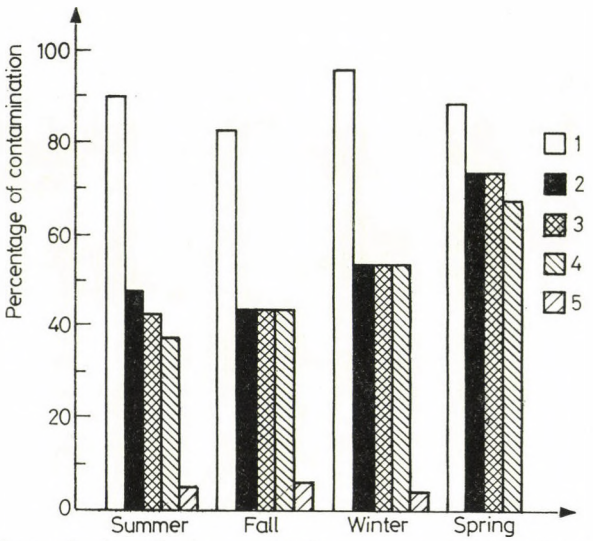

Fig. 3. Share of ochratoxin A-producing moulds in mycopopulations isolated from dairy cattle feeds during the third year of study. 1: moulds; 2: ochratoxin-producing moulds; 3: *Penicillium* spp.; 4: *P. verrucosum* var. *cyclopium*; 5: *Aspergillus ochraceus* group

Table 1

Number of feed samples contaminated with OA-producing moulds and OA

Research period	No. of feed samples	No. of feed samples contaminated with moulds	No. of feed samples contaminated with OA-producing moulds	No. of feed samples contaminated with OA/OA-producing moulds	Concentrations of OA $\mu g \cdot kg^{-1}$
First research year	S 22	20	9	O/O	—[a]
	F 17	14	9	4/2	trace — 92.25
	W 19	18	11	13/8	12.50 — 120.00
	Sp 18	15	10	1/1	400.00
Second research year	S 16	16	15	3/3	100.00
	F 19	18	10	4/2	45.00 — 80.00
	W 19	18	8	7/1	16.00
	Sp 23	23	11	4/2	12.00
Third research year	S 21	19	10	11/5	40.00 — 200.00
	F 18	15	8	4/3	52.00 — 80.00
	W 24	23	13	5/3	16.00 — 80.00
	Sp 19	17	14	5/2	16.00 — 40.00

S — Summer; F — Fall; W — Winter; Sp — Spring; [a] not detected

Table 2

Types of feeds contaminated with ochratoxin A

Feed	No. of feed samples tested	No. of feed samples contaminated with OA	Percentage of samples contaminated with OA
Hay	12	10	83
Dried alfalfa	36	9	25
Fresh alfalfa	5	2	40
Concentrate	53	12	23
Pelleted sugar beet pulp	36	8	22
Corn silage	55	20	36

the summer period of the second research year. In that period as much as 94% of the feed samples was contaminated with *Penicillium* spp., which are known as producers of ochratoxin A (Škrinjar, 1985; El-Banna et al., 1987; Pitt, 1987; Macgeorge and Mantle, 1990). The commonest species was *P. verrucosum* var. *cyclopium*.

Mycotoxicological analysis showed that ochratoxin A was found in all seasons, except in the summer period of the first year of study. The concentrations of ochratoxin A varied from hardly detectable (trace) to 400 μg/kg (spring of the first year, Table 1). From toxin-positive feed samples ochratoxin-producing moulds were isolated, too.

Different types of feeds were used for cattle feeding on the farms. It was found out that ochratoxin A consistently occurred in the same types of feeds. Table 2 shows that as much as 83% of the hay samples tested was contaminated with this toxin.

The results obtained in these experiments are consistent with those reported by other researchers (Vengust et al., 1983; Samson and van Reenen-Hoekstra, 1988), who pointed out that grains and other cellulosic materials are optimal substrates for the production of mycotoxins.

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SPHAEROSPORA INFECTION OF AMERICAN CATFISHES *(ICTALURUS PUNCTATUS* AND *I. NEBULOSUS)* IN EUROPE

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The occurrence in Europe of a *Sphaerospora* species described in North America is reported. Based upon its morphological characteristics, the parasite could be identified with the species *S. hankai* described from brown bullhead in Canada. This parasite was found to infect channel catfish *(Ictalurus punctatus)* cultured in farm ponds in Italy and brown bullhead *(Ictalurus nebulosus)* living in the supply channels of fish ponds in Hungary. The spores and sporogonic developmental stages were situated in the lumen of the renal tubules. In the authors' opinion, *S. ictaluri* described from channel catfish can be considered synonymous with *S. hankai*.

Keywords: Catfish, Myxosporea, new species, *Sphaerospora hankai*

After having recognized the substantial pathogenicity and complex development of *Sphaerospora* species of cyprinids (Molnár, 1979, 1980; Csaba et al., 1984; Lom et al., 1985; Baska and Molnár, 1988), the attention of fish parasitologists increasingly turned towards sphaerospores infecting other groups of fish. As a result of this increased interest, new *Sphaerospora* species have been described from marine and freshwater percids *(Dicentrarchus, Epinephelus, Gymnocephalus)*, salmonids *(Onchorhynchus, Salmo)*, long nose skate *(Raja rhina)*, sterlet *(Acipenser ruthenus)*, and even from American catfishes (Sitja-Bobadilla and Alvarez-Pellitero, 1990; Supamattaya et al., 1991; Molnár, 1991; Kent and Hedrick, 1985; Fischer-Scherl et al., 1986; Arthur and Lom, 1985; Baska, 1990; Lom et al., 1989; Hedrick et al., 1990). In the same period, the blood parasites described from common carp by Csaba (1976) were demonstrated first in numerous other cyprinids and then in *Gasterosteus aculeatus, Ictalurus punctatus, Epinephelus malabaricus* and *Gymnocephalus schraetzer*. By now it has become an unquestionable fact that these blood protozoans can be considered the presporogonic stages of sphaerospores circulating in the blood.

This paper reports on the occurrence of a *Sphaerospora* species, hitherto known only from North America, in channel catfish and brown bullhead cultured in Europe. In addition, the synonymization of *Sphaerospora ictaluri* with *S. hankai* is proposed.

Materials and methods

The studies were conducted in Italy and in Hungary in 1991. In Italy, one-year-old, 11—13 cm long specimens of channel catfish *(Ictalurus punctatus)* were obtained from a fish farm close to Udine. One moribund fish and four symptomless specimens were transported to the laboratory in June 1991. In Hungary, brown bullhead *(Ictalurus nebulosus)* commonly occurring in the supply channels of fish ponds were examined. Seven 2–3 months old, 4–5 cm long and fourteen 1- or 2-summer-old, 15–22 cm long brown bullhead specimens, collected from channels of a fish farm east of the River Tisza, were examined in the summer months.

After killing the fish, blood smears were prepared and stained by Giemsa. Small pieces excised from the kidney of the fish were placed under a coverslip and examined as fresh preparations by light microscopy for the presence of *Sphaerospora* spores. If the kidney of a fish was found infected, the part not used for making squash preparations was fixed in Bouin's solution, embedded in paraffin, sectioned, and the sections were stained with haematoxylin and eosin.

Results

Two out of the five channel catfish specimens examined in Italy were found to have spores in the kidney. Infection of high intensity was found, however, only in the fish specimen caught in moribund state. In that fish, *Sphaerospora* spores situated in typical monosporous pseudoplasmodia were demonstrable in the lumen of the renal tubules, accompanied by some young pseudoplasmodia. In the other infected fish specimen only a few scattered spores were observed. In both shape and size, the spores fully conformed to the criteria specified by Lom et al. (1989) for the species *Sphaerospora hankai* Lom, Desser and Dyková, 1989.

Of the brown bullhead specimens examined in Hungary, only the fry proved to be infected. Five specimens had low-intensity while one showed pronounced *Sphaerospora* infection of the lumen of renal tubules (Fig. 1). The spores (Fig. 2) corresponded to the characteristics described by Lom et al. (1989) in both shape and size.

No presporogonic stages were found in the blood smears.

Discussion

It seems that the complex intra- and extra-piscine development of myxosporeans does not set a bar against the transcontinental spread of *Sphaerospora* species. The parasites of American catfishes must have got through to Europe with the introduced fish species. Besides the cases observed by us in Italy and in Hungary, Moshu (1991) reported *Sphaerospora* infection of *Ictalurus punctatus* from Moldavia, and identified the parasite found with the species *S. hankai* Lom, Desser and Dyková, 1989. We also regard the sphaerospores found in channel catfish and brown bullhead as parasite specimens belonging to the very same species which we identify, based upon the priority, with the species *S. hankai*. The morphological characteristics and dimensions of *S. ictaluri* described by Hedrick et al. (1990) fall between the limits published by Lom et al. (1989), and the data reported by the former authors on the developmental stages and pathological conditions are a useful complement to the description of *S. hankai*. The demonstration of blood stages in the blood vessels and renal parenchyma of *I. punctatus* by Hedrick et al. (1990) is especially interesting, as these stages correspond to swimbladder forms of the common carp. So far such *Sphaerospora* developmental stages have been found only by Baska and Molnár (1988) in the blood of cyprinids. The failure to detect blood stages in our cases can be attributed to the fact that the sphaerosporosis observed in the fish examined by us corresponded to a late stage of spore formation.

Few data are available on the species-specificity of sphaerospores. They seem to belong to parasites characterized by a relatively strict specificity. However, the experiments of Körting et al. (1990), in which they successfully transmitted the blood stages of *S. renicola* from goldfish to common carp, indicate that closely related fish species may have common *Sphaerospora* parasites. Because of the above facts, we are of the opinion that the *Sphaerospora* spores and developmental stages observed in the two phylogenetically closely related catfishes belong to the same species which we identify with *S. hankai*. For the same reason, we regard the name *S. ictaluri* Hedrick, McDowell and Groff, 1990 as a junior synonym of *S. hankai* Lom, Desser and Dyková, 1989.

It can no longer be decided with certainty whether this American parasite was introduced to Europe with *Ictalurus nebulosus*, a fish that got into Europe by accident, or with *I. punctatus* which was introduced later according to plan. The available data are insufficient for determining the pathogenicity of the parasite either. We share the view of Hedrick et al. (1990) that the K stages circulating in the blood can cause similar pathological processes in channel catfish than do the corresponding stages of *S. renicola* in common carp.

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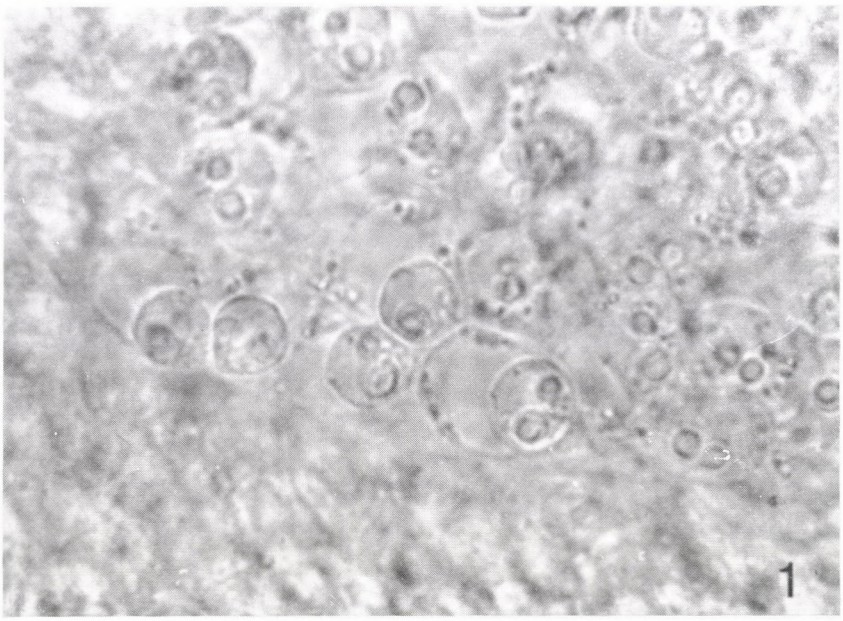

Fig. 1. Spores of *Sphaerospora hankai* in monosporous pseudoplasmodia from the kidney of a brown bullhead fry. Fresh preparation, ×1500

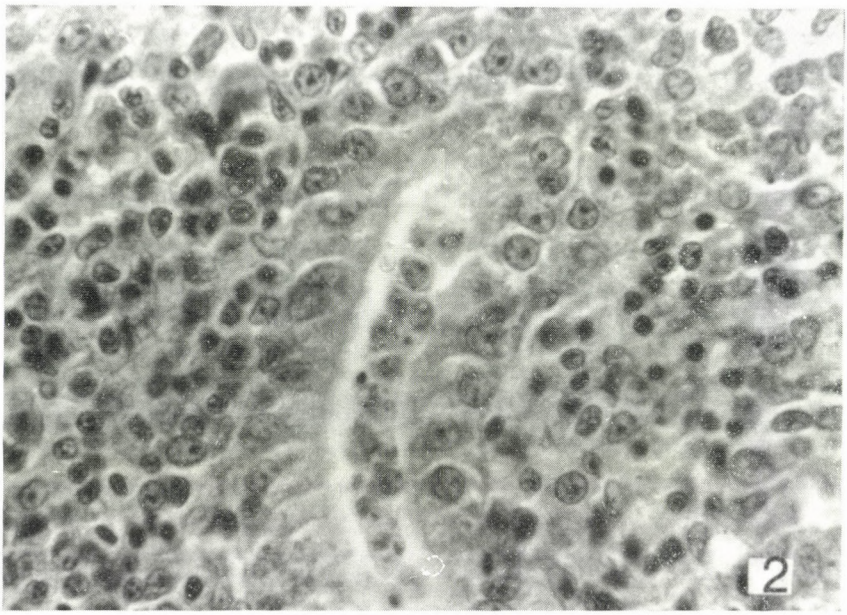

Fig. 2. Pseudoplasmodia and spores in the tubular lumen of the kidney of a brown bullhead fry. Haematoxylin and eosin, ×500

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EARLY DAMAGES TO LUNG CAPILLARIES IN ENZOOTIC PNEUMONIA OF RABBITS

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The present study is part of a series of investigations aimed at characterizing the "enzootic pneumonia" phenomenon in rabbits, both from the clinical-epidemiological and the pathophysiological point of view. All affected rabbits included in this study showed an acute injury to pulmonary microvasculature, similar to changes reported in pulmonary infections caused by Gram-negative bacteria. Similar alterations were not found in the control rabbits. The clinical and morphological findings revealed certain similarities between the Enzootic Pneumonia Syndrome of rabbits and Adult Respiratory Distress Syndrome (ARDS) of man. Therefore, it is suggested that, just as in the human syndrome, heterophils (PMN) are directly involved in the pathogenesis of the process. Many of the similarities noted between the two syndromes would make it possible to postulate the natural disease in rabbits as an eventual animal model suitable for extrapolating data to humans.

Keywords: Early damages, lung capillaries, enzootic pneumonia, rabbit

Pneumonia caused by Gram-negative bacteria is one of the commonest pathologic conditions in rabbits. This is true for both commercially bred and laboratory rabbits (Flatt and Dunworth, 1971*a*; Flatt, 1974; Feinstein and Rehbinder, 1988). *Pasteurella multocida* and, to a lesser extent, *Bordetella bronchiseptica* (Watson et al., 1975) are cited among the most frequently isolated agents. Besides producing pneumonia, *P. multocida* is described as being capable of producing, among other pathologic changes, septicaemia, otitis and rhinitis (Flatt and Dunworth, 1971*a*; Flatt, 1974). The most important clinical signs described until now for pneumonia and septicaemia cases include dyspnoea, polypnoea, marked cyanosis, lack of appetite, and abdominal dilatation due to the accumulation of exudate (Mendoza, 1991).

In pneumonia cases, gross pathologic changes in the lungs are located mainly in the ventral regions and may range from gray nodular foci of different size to large consolidated areas (Flatt and Dunworth, 1971*a*, *b*; Flatt, 1974; Watson, 1975; Feinstein and Rehbinder, 1988; Uzal et al., 1989). The changes found in septicaemia cases are consistent with this.

Pneumonia is usually interstitial in location, presenting marked proliferation and accumulation of alveolar macrophages (PAM) and heterophils in the alveolar capillaries and the presence of varying numbers of heterophils in the alveolar, bronchiolar and/or bronchial lumina (Flatt and Dunworth, 1971*a*, *b*; Flatt, 1974; Watson, 1975).

Materials and methods

Fifteen 2–3 months old New Zealand White rabbits were taken from two farms located in the Bogotá Savanna. Ten rabbits showed clinical signs characteristic of acute pneumonia, including dyspnoea, polypnoea, severe cyanosis of mucous membranes and internal surface of the ears, lack of appetite, and abdominal dilatation. These rabbits served as experimental animals. The remaining 5 clinically healthy rabbits served as the control group.

Sampling. All rabbits were placed under tranquilizers and general anaesthesia with 5 mg/kg xylazine (Rompun®) i.m. and 10 mg/kg ketamine (Ketalar®) i.m., respectively. The ventral area of the neck was shaved, the trachea was exposed, and fixing solution was applied with a 5-ml syringe. The fixing solution used was 5% glutaraldehyde of 4 °C temperature (pH 7.2). This method was used in 5 experimental and 2 control rabbits. The lungs of the remaining rabbits, which were under anaesthesia, were also fixed. Their tracheae and lungs were exposed and then fixed via the larynx to a 25 cm column of fixing solution (glutaraldehyde) in order to obtain better pulmonary expansion. The samples were processed by routine procedures of light microscopy (haematoxylin-eosin and toluidine blue staining) as well as transmission electron microscopy.

Results

All affected animals showed marked abdominal dilatation and cyanosis, especially of the ears. The gross pathologic findings were identical to those seen in septicaemic cases and included severe subcutaneous oedema with severe congestion and cyanosis of all organs. A large volume of fibrinopurulent exudate, containing fibrin threads and often showing sanguinolent discoloration, accumulated in the abdominal and thoracic cavities. The heart of all affected rabbits showed pronounced dilatation of the right auricle.

Morphologic alterations seen in the lungs of the 10 pneumonic animals could be divided into two large groups by their apparent time of evolution. Only the findings observed in the pulmonary microvasculature are described in this paper. The first group contains damages which develop early in the pulmonary microvasculature (congestion, interstitial and intra-alveolar oedema, microthrombus, fibrin in the alveolar lumen, intravascular sequestration of heterophils, migration of heterophils into the alveolar lumen, and haemorrhage). The second group is composed of changes which take a longer time to appear, e.g. hyperplasia of pneumocytes II, increase in the number of alveolar macrophages (PAM), thickening of the interalveolar septa, and activation of lymphoid aggregates). All this is the subject of another publication (Mendoza, 1991).

Table 1

Early damages in lung capillaries in enzootic pneumonia of rabbits

Morphologic alterations	Experimental rabbits										Control rabbits				
	1	2	3	4	5	6	7	8	9	10	1	2	3	4	5
Congestion	*****	*****	**	***	***	*****	*****	***	***	***	*	*	*	*	—
Alveolar oedema	**	*	**	***	***	***	*****	*****	*****	**	—	—	—	—	—
Fibrin in the alveolar lumen	*	*	*	**	*	*	**	*	**	*	—	—	—	—	—
Alveolar haemorrhage	***	**	**	**	***	**	*****	*****	*****	**	*	—	—	—	—
Intravascular sequestration of heterophils	*	***	*	***	***	***	*****	*****	***	***	—	—	—	—	—
Heterophils in alveolar lumen	*	*	*	**	*	**	***	***	*****	*	—	—	—	—	—

—: no lesion; *: mild lesion; **: moderate lesion; ***: severe lesion; *****: grave lesion

Light microscopy findings

Marked congestion was a consistently occurring lesion (Table 1). All animals exhibited oedema of varying degree. This oedema varied in severity and quality also among different regions of the same lung (Fig. 1). Mixed in with the oedema, fibrin threads were found in some alveolar lumina. The extent and severity of haemorrhage also varied by animal. Haemorrhage was found in circumscribed areas within the lungs, especially in areas with severe lung injury (Figs 1 and 2). The presence of microthrombi, the aggregation of platelets in alveolar capillaries, and the sequestration of heterophils (PMN) in blood vessels were more easily observed in semi-thin sections stained with toluidine blue (Figs 2 and 3). Heterophils were also present in the alveolar lumen in some animals and, together with alveolar macrophages, constituted large areas of consolidation (Fig. 4).

Electron-microscopic findings

The changes observed by light microscopy were corroborated by the electronmicroscopic findings. Electron microscopy provided a better insight into some of the morphologic fundaments of capillary injury. A severe hydropic and even vacuolar degeneration in endothelia as well as in pneumocytes I was seen (Figs 5 and 6). Attempted reparation in other cell types was also observed. Oedema formation could be monitored from its first stages in the interstitial space up to fibrin exudation.

Discussion

The indications of injury to pulmonary microvasculature (congestion, oedema, microthrombus, fibrin in alveolar space, intravascular sequestration and extravascular migration of heterophils, and haemorrhage) can be attributed to one of several factors coming from the infection itself, or from the inflammatory response of the organism to the invading agent(s). Many authors agree in pointing out *P. multocida* as the main pathogen which produces morphologic changes in the lungs of rabbits with enzootic pneumonia. Recently, however, Glávits and Magyar (1990) have demonstrated differences in the pathomorphologic changes produced by *P. multocida* and *B. bronchiseptica*, and concluded that the latter can also induce fatal respiratory disease. In our case, not only were we able to isolate *P. multocida* in its pure form but we also classified it as *P. multocida* type 3 according to the guidelines of international reference laboratories (Figueroa and Morales, personal communication). The endotoxins released by *P. multocida* belong to components of bacterial origin that can cause direct injury to the capillary endothelium (Heddleston and Reber, 1975).

These endotoxins are capable of triggering alterations in the microvascular endothelium. This may result in a highly complex pulmonary reaction the initial stages of which are characterized by acute haemodynamic changes due to an alteration in blood viscosity and a delay in blood flow (Brigham and Meyrick, 1986; Meyrick, 1986; Worthen and Haslet, 1986; Slocombe and Derksen, 1989).

Heterophils (neutrophils) constitute another element potentially relevant in the development of microvascular lesions. Many authors have proposed intravascular sequestration of these cells as the mechanism responsible for directly triggering many functional and morphologic alterations of capillary endothelia (Shasby et al., 1982; Martin, 1984; Meyrick, 1986; Boxer et al., 1990). In humans, intravascular sequestration and migration of neutrophils into the alveolar lumen have been widely studied throughout the course of the Adult Respiratory Distress Syndrome (ARDS), a disease clinically manifested in severe acute dyspnoea, tachypnoea, cyanosis refractory to oxygen treatment, decreased compliance of the respiratory system, and diffuse alveolar infiltration as determined by radiological examination. Morphologically, the main histological features of the human syndrome are – among other changes – alveolar proteinaceous oedema, presence of hyaline membranes, and intra-alveolar haemorrhage (Ognibene et al., 1986; Fowler et al., 1987; Murray et al., 1988).

Although the clinical form of the Enzootic Pneumonia Syndrome has not been sufficiently characterized yet, some resemblances can be demonstrated between this syndrome and human ARDS. The signs consistently present in the examined rabbits were dyspnoea, polypnoea and severe cyanosis. Morphologically even greater resemblances were observed. As shown in Table 1, the changes regarded as vascular phenomena of very early evolution (congestion, oedema, microthrombus formation, fibrin in the alveolar space, haemorrhage, intravascular sequestration and extravascular migration of heterophils) correspond to a great extent to what has been reported in the literature as histological alterations found in ARDS patients (Fowler et al., 1987; Murray et al., 1988; Boxer et al., 1990).

In ARDS, neutrophils seem to be directly involved in the pathophysiology of the process. According to what has been demonstrated, intravascular activation or experimental instillation of complement plays an important role in the attraction and sequestration of heterophils (PMN) in pulmonary capillaries (Webster et al., 1982; Till and Ward, 1986; Boxer et al., 1990). Induction of neutrophil sequestration in animal models e.g. by methods such as (1) the use of bacterial endotoxin (Meyrick, 1986) and (2) phorbol ester infusion (Shasby et al., 1982) was reported to cause an increase of varying degree in lung vascular permeability (Shasby et al., 1982; Meyrick, 1986). Several studies have documented the fact that neutrophils, through their oxidizing products, can be lethal to pulmonary endothelial cells (Martin, 1984; Brigham and Meyrick, 1986).

Experiments with rabbit heterophils indicate that O_2 radicals released by these cells contribute to the production of acute pulmonary oedema rich in proteins in rabbits with normal heterophil counts but not in those showing granulocytopenia (Shasby et al., 1982).

The ultrastructural changes ranging from the development of vacuoles and self-digestive vacuoles in the endothelium and increased quantity of rough endoplasmic reticulum to the presence of perivascular and intra-alveolar oedema confirm vascular damage in this study. This falls in line with reported alterations in endothelial cells caused by neutrophils through a hydrogen-peroxide-dependent mechanism (Martin, 1984).

The number of heterophils in the alveolar lumen was apparently higher in animals which were more severely affected (Fig. 4, Table 1). Migration of such cells into the alveolar lumen may be interpreted as an effect produced by chemotactic activity. Alveolar macrophages may have a direct chemotactic effect through cytokines (mainly IL-1) and other small peptides (fMLP) (Hunninghake, 1980; Harmsen, 1988; Said, 1989).

Murray et al. (1988) have recently commented on the inconvenience of using experimental animal models in order to comprehend the human ARDS phenomenon. The clinical signs described here, the morphologic findings reported up to date, as well as prolonged clinical observations (unpublished data) give room to suggest a more thorough observation of the Enzootic Pneumonia Syndrome in rabbits as an eventual natural animal model of ARDS.

Acknowledgements

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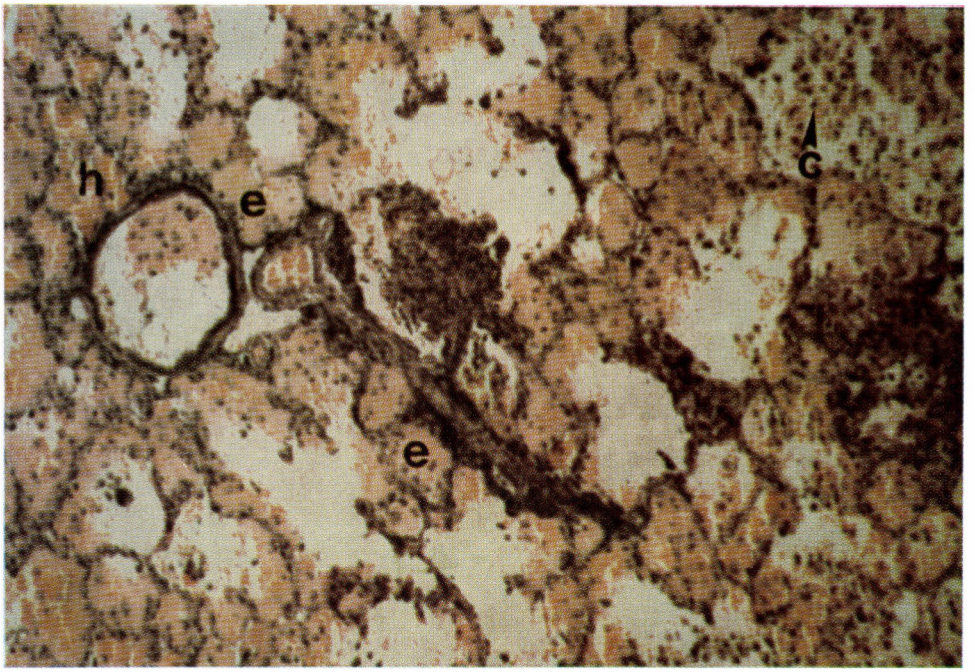

Fig. 1. Severe accumulation of dense alveolar proteinaceous oedema (e). Mixed in with the oedema there is an evident severe haemorrhage (h). Note an increase in the number of alveolar cells (c) possibly of inflammatory origin. Approx. ×100

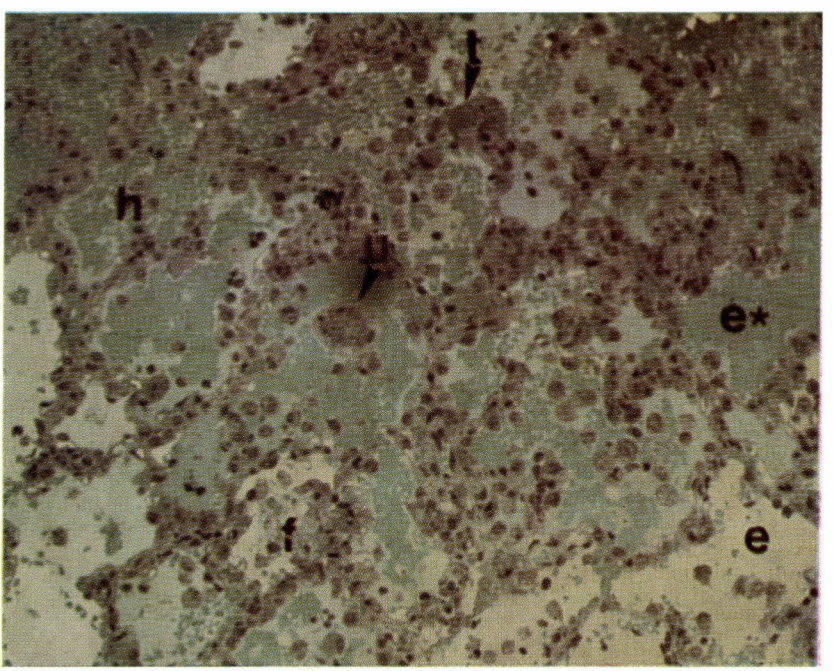

Fig. 2. Massive alveolar haemorrhage (h) together with oedema of variable protein content. Note areas where the intensity of the dye is higher (e*). Microthrombi (t) are more evident by this technique. Intra-alveolar fibrin (f). Different PAM (u) sizes of amorphous contents, possibly of lipidic origin, largely invade the alveolar lumen. Approx. ×200

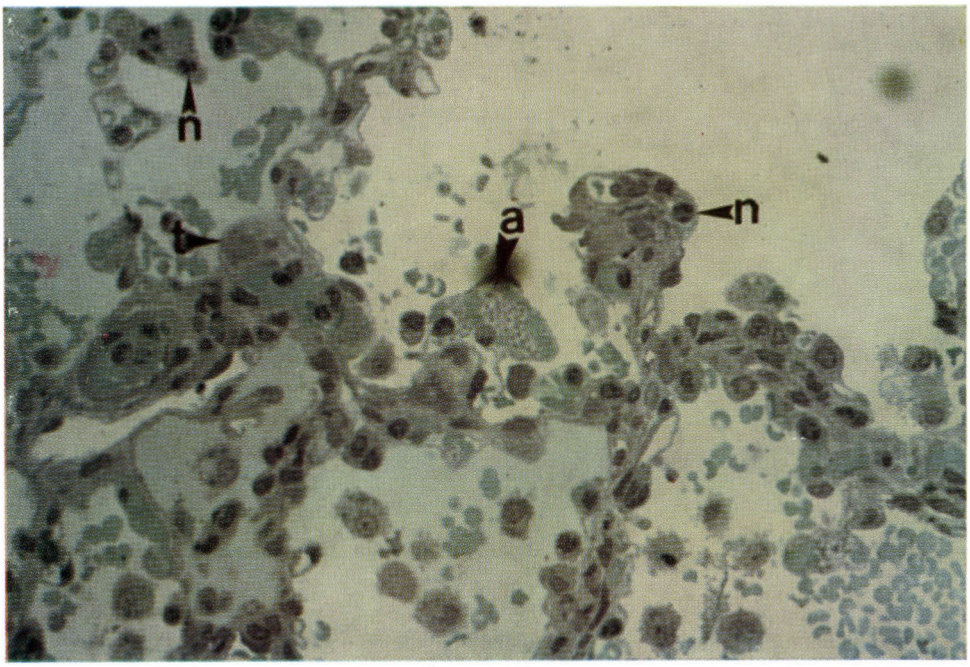

Fig. 3. Fibrin microthrombus (t), platelet aggregation (a) and intravascular sequestration of heterophils (n) are better shown through semi-thin sectioning and toluidine blue staining. Approx. ×200

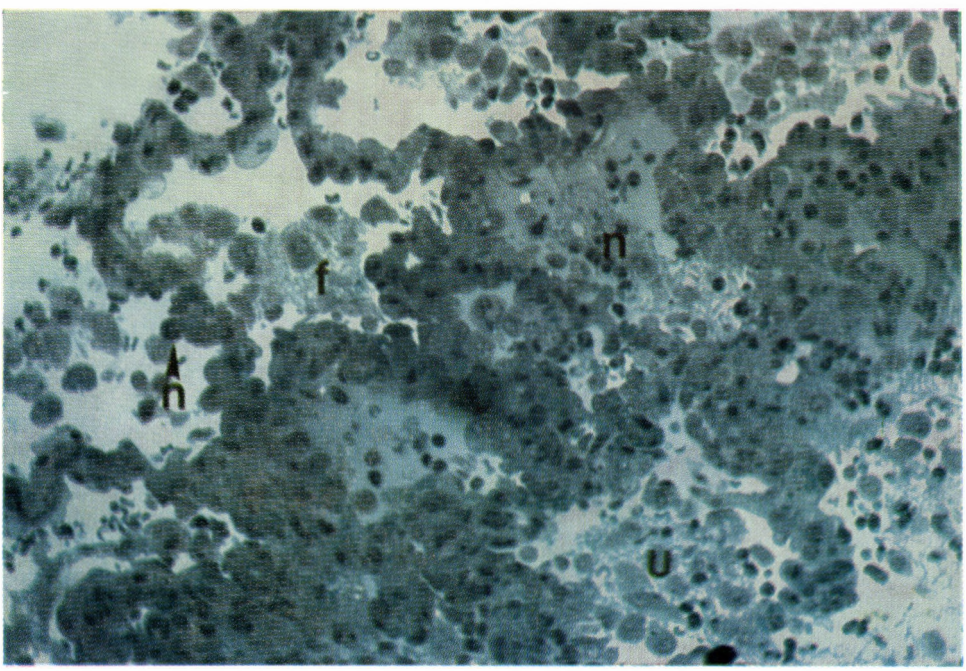

Fig. 4. Severe intra-alveloar heterophil accumulation (n) and intravascular sequestration of the same (arrows, n). Note also fibrin (f) and PAM (u) accumulation in the alveolar lumina. Approx. ×200

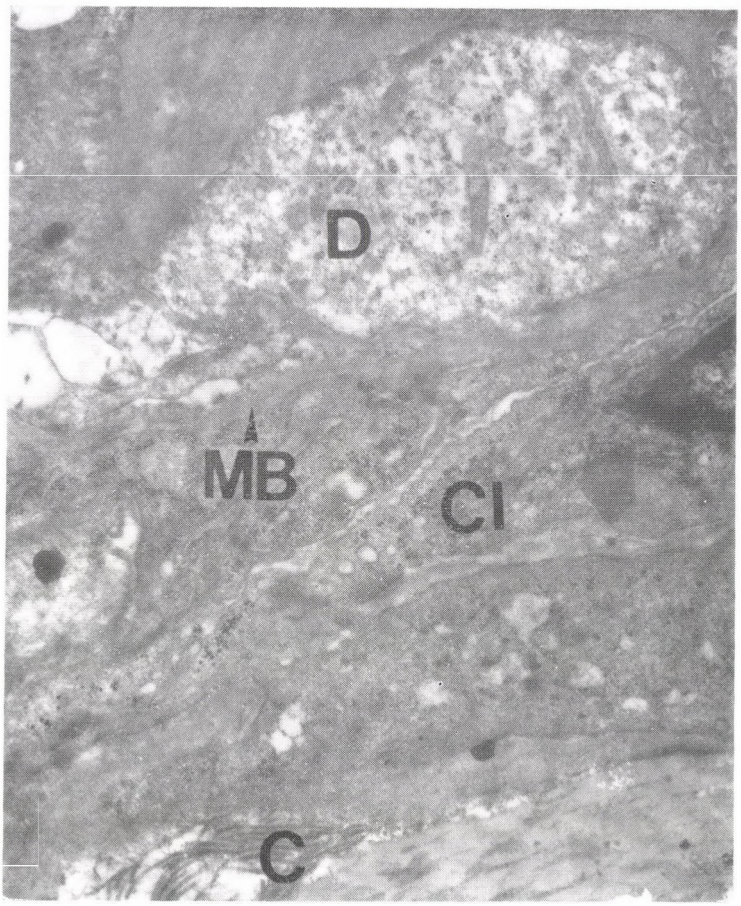

Fig. 5. Capillary endothelium (D). Note size of cytoplasm which has lost electron-density as a sign of degeneration compared to other surrounding cell. Basal membrane (MB), interstitial cell (CI), collagen (C). Approx. ×15,000

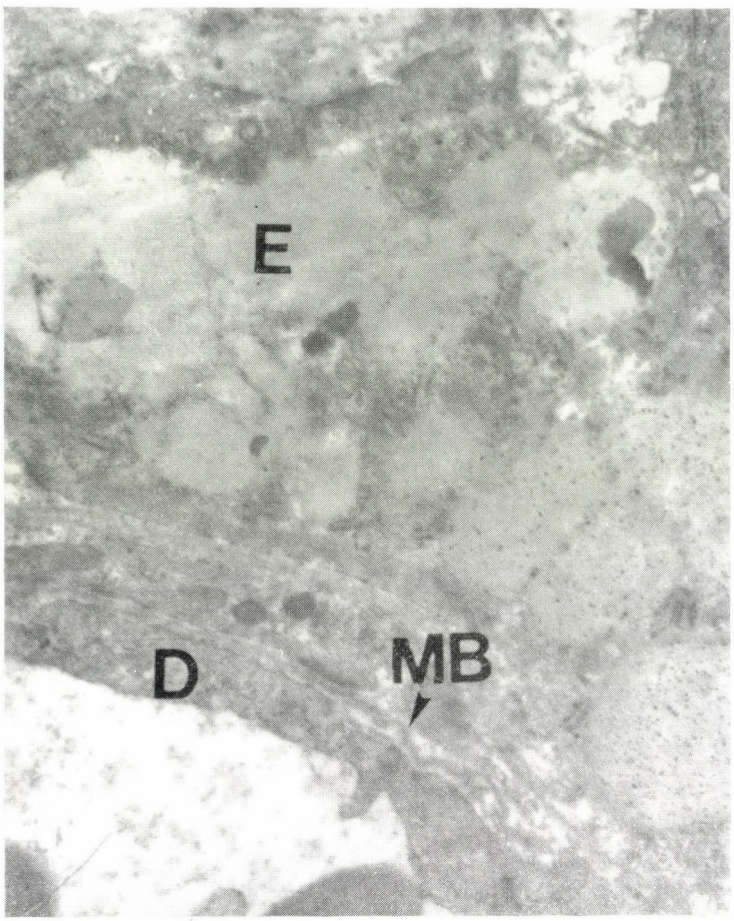

Fig. 6. Subendothelial oedema (E). Endothelial cell cytoplasm (D); basal membrane (MB). Approx. ×12,000

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TERATOLOGICAL EXAMINATION OF THE INSECTICIDE METHYL-PARATHION (WOFATOX 50 EC) ON PHEASANT EMBRYOS 2. BIOCHEMICAL STUDY

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Fertile pheasant's eggs were treated with the insecticide Wofatox 50 EC (50% methyl-parathion) by injection technique on day 12 of the hatching period. Treatment consisted of inoculation of 0.1 ml of different concentrations of the insecticide into the air space of embryonated eggs. The following dose levels were employed: 0.00, 1.35, 13.5 and 135.0 mg/kg egg of active ingredient.

Biochemical changes in the plasma were evaluated by micro (photometric) methods which rendered possible the determination of several blood plasma variables of the embryos. At the highest dose level applied, serum alkaline phosphatase (SAP) enzyme activity and inorganic P concentration of the treated embryos showed statistically differences (reduction) as compared to the control data. Macroscopic alterations were detected at necropsy.

Keywords: Pheasant, embryo, methyl-parathion, biochemistry, teratogenicity

Insecticides used in agricultural practice may exert their toxic influence on wild animals either directly or indirectly. Pheasants are exposed to the effect of chemicals during laying and hatching as their reproductive period coincides with the peak use of some pesticides for plant protection.

This fact justifies the ecotoxicological and, in this case, teratological examination of wild birds. The pheasant is the most important game bird in Hungary. In recent years, the anticholinesterase-type insecticide Wofatox 50 EC has been reported to possess teratogenic potential for bird embryos (Várnagy, 1981*a*; Várnagy et al., 1981; Várnagy et al., 1982). Among these results, the morphological data obtained for pheasant fetuses were demonstrated in detail (Várnagy et al., 1984).

Teratogenicity studies are mainly based on macroscopic findings. In this work some biochemical data (blood plasma variables) obtained by teratological examination of pheasant embryos are reported.

The principal aim of the study was to obtain new information concerning the relationship between morphological and biochemical data. Teratological, mainly biochemical, studies on the effect of Wofatox 50 EC (containing methyl-parathion as active ingredient) on pheasant embryos are presented in this paper.

Materials and methods

Wofatox 50 EC (active substance: 50% methyl-parathion, Chemiekombinat, Bitterfeld, Germany) was used. Fertile eggs collected from our own pheasant *(Phasianus colchicus mongolicus et torquatus)* breed were treated with Wofatox 50 EC on the 12th day of incubation. The insecticide formulation was injected as aqueous emulsion into the air space of eggs (0.1 ml per egg). The following concentrations of Wofatox 50 EC were used: 0.00, 0.02, 0.20 and 2.00%. The middle level of the concentration range corresponded to the concentration employed in the plant protection practice.

Blood samples were withdrawn from pheasant embryos on day 23 of incubation by Várnagy's method (Várnagy, 1981*b*).

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 direct 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) 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.

The incidence of deformity and the mortality rate were analysed for difference from the control by the Chi-square test. The differences between mean embryonic body mass and biochemical variables were evaluated by Student's *t* test (Finney, 1972).

Results

A total of 270 blood and plasma samples obtained from pheasant embryos were examined for three variables. Detailed data are given in Tables 1 and 2.

Mean embryonic body mass showed a statistically significant decrease at the highest dose, and the mortality as well as the number of malformed fetuses were statistically significant at the same dose level.

The biochemical variables of embryos treated with the highest dose level (135.0 mg/kg egg) of Wofatox 50 EC showed a statistically significant decrease as compared to the controls.

Table 1

Principal data of the teratological study of Wofatox 50 EC in pheasant embryos

Dose+ (mg/kg egg)	Embryonated eggs	Body weight of fetuses (g)	Live fetuses	Dead fetuses	Malformed fetuses
0.00	29	15.58±0.90	27	2	3
1.35	32	15.45±0.41	31	1	2
13.50	33	16.21±0.32[d]	33	0	3
135.00	31	14.41±0.75[d]	19	12[d]	19[e]

[d]$P<0.01$; [e]$P<0.001$ vs. control; +active ingredient

Table 2

Summary of plasma biochemical data obtained by teratogenicity study of Wofatox 50 EC on pheasant embryos treated by injection technique on day 12 of incubation

Variables	Control	Dose, mg/kg egg+ 1.35	13.50	135.00
SAP (U/l)	856±122 n=24	821±130 n=26	837±153 n=27	719±134[e] n=15
Ca (mmol/l)	2.74±0.55 n=23	2.72±0.42 n=27	2.72±0.40 n=26	2.64±0.57 n=15
Inorganic P (mmol/l)	2.16±0.52 n=24	2.08±0.65 n=24	2.20±0.48 n=25	1.58±0.36[d] n=14

n = number of samples tested; [d]$P<0.01$; [e]$P<0.001$; +active ingredient

Discussion

The toxicity including teratogenicity of the insecticide Wofatox 50 EC to avian embryos have been tested previously. However, limited information is available on the influence of this pesticide on the pheasant as a non-target organism.

At the highest dose level of the insecticide the morphological and biochemical data showed a very expressive relationship with special regard to the decrease of SAP activity and inorganic P concentration. These changes may contribute to the manifestation of secondary skeletal malformations together with primary hypoplasia or atrophy of the cervical musculature (Várnagy et al., 1984). The rate of embryonic mortality and teratogenicity, as well as changes in the biochemical variables with respect to the control values emphasize the toxic effect of Wofatox 50 EC at that dose.

In conclusion: Wofatox 50 EC, when injected directly into the air space of embryonating pheasant's eggs, caused some malformations (lordoscoliosis, cyllosis) mostly at the highest dose. The changes detected in plasma biochemistry after direct insecticide exposure indicate an expressed intoxication at the highest dose. The middle dose level, corresponding to that recommended for use in plant protection practice, proved to be harmless and untoxic. This fact calls attention to the importance of choosing the right dose, which is a basic point in integrated pest control without constituting a life hazard to this avian species.

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ISOLATION OF CHICKEN ANAEMIA VIRUS FROM BROILER CHICKENS

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Chicken anaemia virus (CAV) infection was demonstrated, by both serology and virus isolation, in 1- to 6-week-old broiler chickens originated from various parent flocks in Hungary. Total losses in the broiler flocks were estimated at 7 to 8%, and about 25% of the chickens failed to reach target body mass by the 7th week of life. The clinical signs, postmortem lesions and histopathological changes of the affected chickens were similar to those of naturally occurring CAV-induced infectious anaemia of young chickens.

In MDCC-MSB1 cell cultures, a chloroform-resistant virus smaller than 50 nm in diameter, resistant to heating at 70 °C for 30 min, and antigenically very closely related to the Cux-1 strain of CAV was isolated from the liver of naturally diseased broilers. This virus isolate was designated the Bia strain of CAV.

Inoculation of susceptible 1-day-old SPF chicks with a CAV-positive liver extract from naturally diseased broilers caused pathological changes characteristic of CAV infection, namely impaired growth, severe anaemia with atrophy of the bone marrow, marked atrophy of the lymphoid organs and petechial haemorrhages throughout the body. A quite similar pathological syndrome was also induced by inoculation of 1-day-old SPF chicks with the MDCC-MSB1 cell-culture-propagated new Bia strain of CAV.

The CAV was successfully reisolated from the livers of experimentally inoculated birds, and antibodies to the reference Cux-1 strain of CAV were also demonstrated by the indirect immunofluorescence test in sera of naturally diseased and experimentally inoculated chickens. No antibodies were found against infectious bursal disease virus, reticuloendotheliosis virus, Marek's disease herpesvirus as well as avian adenoviruses and reoviruses.

The reported disease of young broiler chickens was associated with natural infection of a new isolate of CAV. On the basis of its physicochemical, antigenic and pathogenic characteristics, this virus is similar to other strains of CAV isolated from chickens in other countries.

Keywords: Chicken anaemia virus, isolation, pathogenicity, natural infection

During investigations into a disease of young chickens caused by a contaminated Marek's disease vaccine in Japan, Yuasa et al. (1979) isolated a new pathogen which was resistant to ethyl ether, chloroform, pH 3 and heating at 80 °C for 15 min, and passed through a 25 nm membrane filter. They named it chicken anaemia agent. The true nature of the agent was enigmatic for a long time. It was initially thought to be a parvovirus. However, on the basis of its discovered properties, it later turned out that the agent cannot be assigned to any of the existing virus groups, and Gelderblom et al. (1989) proposed to term it chicken anaemia virus (CAV), a designation which

we adopted in the present study. CAV has a regular icosahedral symmetry and contains a circular single-stranded DNA genome (Gelderblom et al., 1989). It does not replicate in conventional chicken cell cultures, although it propagates well in embryonating chicken eggs (Bülow and Witt, 1986) and in some continuous lymphoblastoid chicken cell lines (Bülow et al., 1985; Yuasa, 1983).

Susceptible day-old SPF chicks inoculated with CAV became severely stunted and depressed with anaemia, and up to 50% of them died within four weeks after inoculation without showing any other specific symptom. Gross and histopathological examinations revealed impaired growth, severe anaemia with atrophy of the bone marrow, marked lymphocytic depletion with atrophy of the lymphoid organs and petechial haemorrhages throughout the body (Bülow et al., 1986*a*; Goryo et al., 1985, 1987, 1989; Lucio et al., 1990; McNulty et al., 1990*a*; Yuasa and Imai, 1986; Yuasa et al., 1979).

Since the initial recognition of CAV, there have been numerous reports on the isolation of the virus from young anaemic chickens (Chettle et al., 1989; Goryo et al., 1985; Lucio et al., 1990; McNulty et al., 1990*a*), from a field isolate of classical Marek's disease virus (MDV) causing an early mortality syndrome (Bülow et al., 1983) and from broiler chickens affected with blue wing disease or anaemia-dermatitis and/or haemorrhagic syndrome (Bülow et al., 1986*a*; Engström, 1988; McNulty et al., 1989*b*; Vielitz and Landgraf, 1988; Yuasa et al., 1983, 1987). No remarkable differences have been recognized in pathogenicity among the various CAV strains isolated so far, and they have also been shown to belong to a single serotype (Bülow et al., 1985; Engström, 1988; Goryo et al., 1987; McNulty et al., 1989*b*, 1990*a*; Yuasa and Imai, 1986).

CAV is widespread in all major chicken-producing countries of the world both in heavy meat-type and light egg-laying chickens of all ages (Bülow et al., 1983; Chettle et al., 1989; Engström, 1988; Goryo et al., 1985; Lucio et al., 1990; McNulty et al., 1988, 1989*b*; Otaki et al., 1987; Yuasa et al., 1983, 1985, 1987), and infection of specific-pathogen-free (SPF) chicken flocks has also been demonstrated (McNulty et al., 1988, 1989*a*; Nicholas et al., 1989, Yuasa et al., 1985). This latter fact is rather important, as CAV is known to be egg transmitted (Yuasa and Yoshida, 1983) and replicates asymptomatically in chicken embryos (Bülow and Witt, 1986). Thus, the danger exists that vaccines prepared using embryos derived from infected SPF flocks could be latently contaminated with the virus, though this has not been demonstrated yet (Nicholas et al., 1989).

Owing to its immunosuppressive effect, CAV depresses vaccinal immunity (Box et al., 1988; Otaki et al., 1988), considerably aggravates several diseases of young chickens, and also increases susceptibility to concurrent or secondary bacterial, viral and fungal infections (Bülow et al., 1983, 1986*a*,

b; Engström et al., 1988; Otaki et al., 1987; Vielitz and Landgraf, 1988; Yuasa et al., 1980).

The present paper reports the isolation of CAV during the course of investigations into an outbreak of the runting/stunting syndrome in young broiler chicken flocks. Pathogenicity of the new CAV strain is also presented.

Materials and methods

Experimental chickens

For experimental inoculations one-day-old SPF White Leghorn chickens were obtained from the flock of Phylaxia Veterinary Biologicals Co. (Budapest, Hungary) which was found to be free of CAV infection by regular serological examinations. The birds were reared in conventional wire pens in animal rooms and were fed a commercial broiler starter diet. Feed and water were available *ad libitum*.

Cell cultures

MDCC-MSB1 T-lymphoblastoid cell line was obtained through the courtesy of Dr. V. von Bülow (Institute of Poultry Diseases, Faculty of Veterinary Medicine, Free University of Berlin, Germany), and was grown and maintained as described by Yuasa (1983). Cultures of chick embryo fibroblasts (CEF) were prepared from 10-day-old SPF White Leghorn chicken embryos as described elsewhere (Drén et al., 1988).

Viruses

The Cux-1 strain of CAV (Bülow et al., 1983) was obtained from Dr. V. von Bülow and the nondefective T-strain of reticuloendotheliosis virus (REV) from Dr. R. L. Witter (Regional Poultry Research Laboratory, East Lansing, U.S.A.). MD7/13 virulent strain of MDV was isolated from a field case (Drén, 1972). Infectious bursal disease virus (IBDV), used for serum neutralisation tests in this study, was also a field isolate adapted to CEF. The nondefective REV-T strain, MD7/13 strain of MDV and IBDV were propagated in CEF cultures, whereas the Cux-1 strain of CAV was grown in MDCC-MSB1 cells.

Immune sera

Chicken anti-CAV-Cux-1 immune serum was kindly provided by Dr. V. von Bülow or was prepared in 4-week-old SPF White Leghorn chickens using the inoculation schedule described by McNulty et al. (1988). Production

of fluorescein-isothiocyanate (FITC)-labelled rabbit antiserum to chicken IgGMA has been described elsewhere (Drén et al., 1988).

Assays for antibodies

Chicken serum samples were tested for antibodies to IBDV by the serum neutralisation test on CEF cultures. Antibodies to avian adenoviruses and reoviruses were assayed by the radial double diffusion gel precipitation plate test using the reagent kits from SPAFAS Inc. (Storrs, U.S.A.). Antibodies to CAV, REV and MDV were detected by the indirect immunofluorescence (IF) technique using respectively as antigen CAV strain Cux-1-infected MDCC-MSB1 cells (Bülow et al., 1985; McNulty et al., 1988; Yuasa et al., 1985), REV strain T-infected and MDV strain MD7/13-infected CEF cells deposited and fixed onto Multitest slides (Flow Laboratories Ltd, Irvine, Scotland). Slides with the fixed cells were stored over silica gel at –20 °C for later use. Uninfected cells, positive and negative serum controls and a serum-free phosphate-buffered (0.01M, pH 7.2) saline conjugate control were included in the assay. The specificity of the assay for each virus was proved by blocking the staining with a surplus of unlabelled rabbit anti-chicken IgGMA immune serum before the addition of the conjugate. All samples were tested by this technique at 1 : 10 and/or 1 : 20 dilutions.

Preparation of liver extract

About 20% (w/v) homogenates were prepared from the livers of naturally diseased and experimentally inoculated chickens in RPMI 1640 medium containing ten times the usual concentration of antibiotics. Homogenates were frozen and thawed three times and centrifuged at 2000 g for 30 min, and the clear supernatants were used for further studies.

Isolation of CAV

Isolation of CAV from liver extracts was carried out in MDCC-MSB1 cells as described by others (Bülow et al., 1985; Yuasa et al., 1983) using 24-well tissue culture plates (Flow Laboratories Ltd, Irvine, Scotland). From each specimen 100 μl aliquots were inoculated into each of two culture wells containing 1 ml MDCC-MSB1 cells, seeded at 3 to 5×10^5 cells ml^{-1} in RPMI 1640 medium supplemented with 10% (v/v) fetal bovine serum (kindly provided by Dr. G. F. de Boer, Central Veterinary Institute, Lelystad, the Netherlands), 10% (w/v) tryptose phosphate broth (Difco Laboratories, Detroit, U.S.A.), 200 international units ml^{-1} penicillin G potassium, 200 $\mu g\ ml^{-1}$ streptomycin sulphate, 120 $\mu g\ ml^{-1}$ gentamycin sulphate and 2.5 $\mu g\ ml^{-1}$

amphotericin B (fungizone). Control cultures remained uninoculated. Cultures were incubated at 39 to 40 °C in an atmosphere of 5% (v/v) CO_2, and were subcultured at 2- to 3-day intervals by transferring 200 μl cell suspension into 1 ml warm fresh growth medium. Cultures were examined daily for cytopathic effect, which was defined as failure of the cells to grow (Yuasa et al., 1983). Cultures showing cytopathic effect were harvested and examined for the presence of CAV antigens by the indirect IF technique using chicken anti-CAV-Cux-1 immune serum and FITC-labelled rabbit anti-chicken IgGMA immune serum. Uninoculated cultures served as negative controls and those infected with the Cux-1 strain of CAV were used as positive controls for the indirect IF assay. Granular, bright-green intranuclear fluorescence, in the absence of reactions in uninfected culture cells, was considered specific for CAV antigens.

Titration of CAV in MDCC-MSB1 cells

CAV titrations were performed in MDCC-MSB1 cell cultures by the method of G. F. de Boer (personal communication) using 96-well tissue culture plates (Flow Laboratories Ltd, Irvine, Scotland). Serial twofold dilutions of the sample were made beginning with 1 : 20 dilution, and each dilution was tested in six cultures with six passages. Infectivity endpoints were based on cytopathic effect. Median tissue culture infective doses ($TCID_{50}$) were calculated according to Reed and Muench (1938).

Filtration and test for sensitivity to chloroform

CAV-positive liver extracts were filtered sequentially through 1.2, 0.8, 0.45, 0.22 and 0.05 μm pore-size membranes (Millipore Corp., Bedford, U.S.A.) which had previously been saturated with newborn calf serum. The final filtrate was mixed with 7.5% (v/v) chloroform, shaken intermittently and incubated for 10 min at room temperature. The mixture was centrifuged at 2000 g for 10 min and the resultant aqueous fraction was assessed for the presence of CAV by titration in MDCC-MSB1 cells.

Heat treatment of CAV isolate

Equal volumes of the stock virus suspension were placed in each of four tubes in an ice-bath. Three tubes were respectively incubated at 70 °C for 5 min and 30 min and at 80 °C for 15 min in a water bath and then returned to the ice-bath. The infectivity of the virus in all four tubes was subsequently titrated in MDCC-MSB1 cells. The Cux-1 strain of CAV was used as a reference for this study.

Haematocrit

Haematocrit values were determined by the micromethod using 50-mm-long standard heparinized capillary tubes (OMKER, Budapest, Hungary). Triplicate measurements were made from each bird on all occasions. Birds with haematocrit levels below 27% were deemed anaemic (Yuasa et al., 1979).

Histopathology

Following postmortem examination, tissue samples were collected, fixed in 10% (v/v) buffered neutral formaldehyde solution and processed by standard paraffin techniques. Sections were stained with haematoxylin and eosin.

Experimental inoculation

Experiment 1. Five 1-day-old SPF chicks were inoculated intramuscularly into each leg with 1 ml of CAV-positive native liver extract from naturally diseased broilers. CAV infectivity titre of the inoculum was not determined by titration. Four control chicks were left untreated and housed in a separate room.

Experiment 2. Five 1-day-old SPF chickens were inoculated intramuscularly with a filtered and chloroform-treated CAV-positive liver extract from naturally diseased broilers at a dose of $10^{3.07}$ $TCID_{50}$ of CAV per chick. Four uninoculated chicks were kept separately and served as control.

Experiment 3. Fifteen 1-day-old SPF chickens were divided into three equal groups. Five chicks were inoculated intraperitoneally and/or intramuscularly at 1-day-old each with $10^{6.9}$ $TCID_{50}$ of the new Bia strain of CAV as cell-free supernatant fluid from the 10th passage in MDCC-MSB1 cells. Five chickens were contact exposed from one day of age by intermingling and housing together with the inoculated birds. The third group of five chickens was kept separately to serve as isolated controls.

In each experiment, all birds were weighed at 7-day intervals, and the biological body mass gains was calculated for each individual bird according to the formula published by Harris (1966). At 7, 14, 21 and 28 days of age all survivors were bled into heparinized microhaematocrit capillary tubes for haematocrit determinations, and serum samples were collected for serological examinations. The experiments were terminated when the chickens were 28 days old.

All birds dying during the experiments or killed at the end were necropsied, the gross lesions were recorded, and tissue samples were collected for histological examination.

Statistical analysis of the biological body mass gain and haematocrit values of the infected and control groups of animals was conducted by Student's *t* test.

Results

On a broiler breeding farm, an increasing number of retarded and anaemic birds was noticed after 6 to 10 days of age. The proportion of affected chicks varied between 15 and 20% among flocks. On the farm there were three houses, each accommodating 1000 chicks. The houses were restocked at 2- to 3-week intervals with day-old chicks obtained from various commercial hatcheries. Thus, broilers of different ages and derived from different parent flocks were present simultaneously on the farm, but in each house only penmates were reared. Total mortality of the flocks was estimated at 7 to 8%, but about 25% of the chickens failed to reach target body mass by the 7th week of life.

At field observation of three 1-, 3- and 6-week-old flocks, the affected chicks were listless, reluctant to move, and stood with closed eyes and drooped wings. Their feathers were ruffled, and the development of primary feathers was delayed and irregular. The whole body of the affected chicks was more or less pale. These chicks were markedly smaller than their unaffected penmates, and they huddled together causing patchy damp litter.

At necropsy of dead and sacrified ailing chickens, small greyish-white foci were found in the liver and the spleen. The wall of the proventriculus was thickened, and petechial haemorrhages and caseous necrotic depositions were present on its mucous lining. The thymus lobes and the bursa of Fabricius of the sick chicks were substantially smaller in size than those of age-matched healthy chicks. No other gross lesions were found.

For detailed laboratory investigations seven diseased chicks were selected randomly. Histological examination revealed inflammatory infiltrations and proliferations consisting mainly of lymphocytes and histiocytes in the liver and the spleen. There was marked depletion of lymphocytes in the thymic cortex and in the follicles of the bursa of Fabricius. Their lymphoreticular stroma was, however, normal. Sometimes haemorrhages and fibrosis occurred in the interstitial tissue. Lymphocytes and histiocytes infiltrated the superficial and deep glands in the proventriculus, and proliferation of the glandular tissue was occasionally seen. In one bird, heterophil granulocytic endoneural infiltration of the peripheral nerves was found.

Antibodies to the Cux-1 strain of CAV were demonstrated in the sera of two out of seven chicks tested by the indirect IF method. No antibodies were found against IBDV, REV, MDV as well as avian adenoviruses and reoviruses.

Virus isolation in MDCC-MSB1 cells

Virus isolation attempts were carried out on MDCC-MSB1 cell cultures from both the native and the filtered chloroform-treated liver extract of naturally diseased broilers.

In cell cultures inoculated with the native liver extract, a cytopathic effect appeared after three blind passages. The cells became swollen after about 30 hours of incubation and the nuclei contained small vacuoles and assemblies of chromatin as described for CAV by Bülow et al. (1985). On further incubation, most of the cells became disrupted. In the cells of these cultures a granular, bright-green intranuclear fluorescence occurred on indirect IF staining with the reference anti-CAV-Cux-1 chicken immune serum. The immunofluorescence appeared as variable irregularly shaped granules scattered all over the enlarged cells. Occasionally large ring-shaped fluorescent structures were observed in some cells. No such reactions were seen in the cells of uninoculated control cultures.

Following inoculation of MDCC-MSB1 cells with the filtered and chloroform-treated liver extract, a cytopathic effect was observed after four subcultures of the cells. On indirect IF staining of these cell cultures with the reference anti-CAV-Cux-1 chicken immune serum, the infected cells showed an intranuclear fluorescent pattern completely identical to that described above (Fig. 1).

This latter virus isolate, which has been designated the Bia strain of CAV, was serially propagated in MDCC-MSB1 cell cultures and was chosen for further characterization studies. It grew readily in MDCC-MSB1 cell cultures with a characteristic cytopathology as described in the preceding paragraph. The fluorescence in infected cells stained with the reference anti-CAV-Cux-1 chicken immune serum by the indirect IF method was indistinguishable from that in CAV strain Cux-1-infected cells. A similar intranuclear fluorescence was also observed when sera from convalescent chickens experimentally inoculated with the new Bia strain were used for indirect IF staining of CAV strain Cux-1-infected MDCC-MSB1 cells.

Thermal stability of the Bia strain was comparable to that of the Cux-1 strain of CAV used as a reference (Table 1).

Experimental inoculation

For reproduction of the naturally occurring syndrome and to test the pathogenicity of the new CAV isolate, susceptible day-old SPF chicks were inoculated in three separate experiments with a native and a filtered chloroform-treated CAV-positive liver extract from naturally diseased broilers as well as with the tissue-culture-propagated Bia strain of CAV.

Table 1

Heat resistance of chicken anaemia virus (CAV) strains

Treatment	CAV strains	
	Bia	Cux—1
70 °C for 5 min	3.66[a]	3.59
70 °C for 30 min	1.30	1.59
80 °C for 15 min	—	—
No (control)	5.59	5.39

[a]$\log_{10}$ $TCID_{50}$ titre. Titration was performed in MDCC-MSB1 cells using six cultures per virus dilution and six passages

Table 2

Response of chickens to inoculation at 1-day-old with chicken anaemia virus (CAV)-positive liver extract from naturally diseased broilers

Age in days	Group	No. chicks died	Body mass (g) mean±SD	BBMG[e] mean±SD	Haematocrit % mean±SD (range)	Anti-CAV antibody
1	Inoculated n = 5		37.2± 2.9			
	Control n = 4		37.5± 3.1			
7	Inoculated		48.6± 6.6	1.83±1.01	30.2±3 (27—33)	0/5[g]
	Control		53.2± 7.9	2.63±1.04	32.0±0.8 (31—33)	0/4
14	Inoculated	1[a]	60.2±10.6	1.84±0.80[f]	26.5±8.4 (14—32)	0/4
	Control	1[b]	84.2±16.1	3.88±0.84	33.2±3.3 (31—37)	0/4
21	Inoculated	2[c]	91.5±24.7	2.85±1.34	29.0±0	0/2
	Control		120.3±15	4.86±0.79	32.0±1 (31—33)	0/3
28	Inoculated	1[d]			29.0±0	1/1
	Control				33.3±2.9 (30—35)	0/3

[a]Died at 13-day-old; [b]Killed at 14-day-old; [c]Died at 14 and 16 days of age; [d]Died at 25-day-old; [e]Biological body mass gain; [f]$p < 0.02$ compared with control; [g]Number positive over number tested

Experiment 1. Results of inoculation with the native liver extract are recorded in Table 2. Inoculated chicks became severely anaemic, stunted and lethargic, and four of them died between 13 and 25 days of age without showing any other specific symptom before death. They were seriously retarded in growth: mean biological body mass gain of the inoculated group was significantly lower than that of the control group at 14 days of age.

At necropsy of the carcases, hepatic dystrophy and signs of anaemia such as light red watery blood, noticeable pallor of the skin, musculature and internal organs were found. The thymus glands were severely atrophied. They had regressed to a size hardly visible to the naked eye and often contained small haemorrhages. The bursa of Fabricius was also only about half its normal size and sometimes discrete haemorrhages occurred on its epithelial and serous lining (Fig. 2). In the majority of birds, the bone marrow was discoloured and pale, and occasionally became entirely light flesh-to-yellow-coloured (Fig. 3). Numerous ecchymotic and petechial haemorrhages occurred throughout the skeletal muscles and subcutaneous tissue, and were also noted along the coronary artery in one bird.

Histological lesions were found regularly in the bone marrow and thymus and rarely in other lymphoid organs. The most striking changes were seen in the bone marrow and thymus. In the bone marrow, there was severe hypoplasia, accompanied by depletion of cells of both the erythrocytic and the granulocytic series, with haematopoietic cells being replaced by fatty tissue, and with no evidence of erythropoiesis in the most serious cases (Fig. 4). The thymic cortex was more or less depleted of lymphoid elements. The cortex became thinner, and occasionally thymocytes formed only a few layers in the subcapsular areas of the cortex. In the most serious cases, there was complete loss of architecture with no distinction between the cortex and the medulla of the thymus, and sometimes solely the reticular stroma of the organ remained (Fig. 5). Atrophy of follicles in the bursa of Fabricius was observed less frequently, and only a variable proportion of the lymphoid follicles was usually affected, while the cellular morphology of all the other follicles seemed to be normal. The affected follicles were more or less depleted of lymphocytes, and occasionally the interstitial tissue was only seen (Fig. 6). Diffuse necrosis was found in the bursa of one bird. In the spleen, the thymus-dependent periarteriolar lymphocyte sheats were moderately depleted of lymphocytes, whereas the cellularity of the bursa-dependent lymphoid follicles (germinal centres) and the periellipsoidal lymphoid tissue was normal in appearance.

The single surviving chick in the inoculated group had no gross lesions when killed at the end of the experiment. Histological examination revealed hypoplasia of the bone marrow and scattered foci of proliferating myelopoietic cells in the liver. Its serum was positive for antibodies to CAV.

No antibodies to IBDV, REV, MDV as well as avian adenoviruses and reoviruses could be demonstrated in serum samples collected 7 to 28 days after inoculation.

CAV with the same features as the field isolate was readily reisolated in MDCC-MSB1 cell cultures from both the native and the filtered chloroform-treated liver extract of those two chicks that died 13 and 14 days after inoculation.

Experiment 2. Chickens inoculated with the filtered and chloroform-treated liver extract from naturally diseased broilers showed no disease (Table 3). Only the haematocrit values of two chicks dropped close to the pathological level 14 to 21 days after inoculation, and the mean biological body mass gain of the inoculated chicks reached only about 75% of that of the controls at 28 days of age. This difference was, however, statistically not significant. Four of five chicks had developed antibody to CAV by 28 days after inoculation.

Experiment 3. Results of inoculation with the cell-culture-propagated Bia strain are given in Table 4. Inoculated chicks became emaciated and

Table 3

Response of chickens to inoculation at 1-day-old with a filtered and chloroform-treated chicken anaemia virus (CAV)-positive liver extract from naturally diseased broilers

Age in days	Group	Body mass (g) mean±SD	BBMG[b] mean±SD	Haematocrit % mean±SD (range)	Anti-CAV antibody
1	Inoculated[a] n = 5	37.0± 4.0			
	Control n = 4	40.2± 5.2			
7	Inoculated	51.8±10.2	2.46±1.01	33.2±1.6 (32—36)	
	Control	50.5± 5.9	1.64±0.31	33.0±0.8 (32—34)	
14	Inoculated	81.0±18.7	3.67±1.13	31.0±2.2 (28—34)	
	Control	85.7±11.9	3.61±0.32	33.0±1.1 (32—34)	
21	Inoculated	120.4±29.9	4.65±1.34	33.4±3.3 (29—38)	3/5[c]
	Control	132.5±22.2	4.87±0.51	33.2±0.9 (31—33)	0/4
28	Inoculated	154.2±36.2	4.92±1.21	34.4±2.2 (32—38)	4/5
	Control	206.0±45.7	6.53±1.07	32.5±1.3 (31—34)	0/4

[a]$10^{3.07}$ $TCID_{50}$ CAV-containing liver extract per chick; [b]Biological body mass gain; [c]Number positive over number tested

Table 4

Response of chickens to inoculation at 1-day-old with a chicken anaemia virus (CAV) isolated from naturally diseased broilers in MDCC-MSBl cell cultures

Age in days	Group	No. chicks died	Body mass (g) mean±SD	BBMC[g] mean±SD	Haematocrit % mean±SD (range)	Anti-CAV antibody
1	Inoculated[a] n = 5		40.4± 5.0			
	In-contact n = 5		39.4± 4.8			
	Control n = 5		36.0± 4.1			
7	Inoculated	1[b]	60.0± 5.0	3.15±0.85	33.6±2.97 (30—38)	
	In-contact		56.2± 8.0	2.75±1.16	32.6±2.4 (30—36)	
	Control		58.4± 7.1	3.87±0.50	35.6±2.6 (32—38)	
14	Inoculated	1[c]	94.3± 4.6	4.08±0.38	21.0±5.9[f] (13—27)	3/4[h]
	In-contact	1[d]	96.6±18.4	4.60±1.02	30.6±1.5 (28—32)	0/5
	Control		100.4±12.4	5.56±0.52	31.6±2.7 (29—36)	0/5
21	Inoculated		141.3± 6.4	5.19±0.64[e]	34.3±1.5 (33—36)	3/3
	In-contact		157.0±31.9	6.10±1.42	31.8±1.7 (30—34)	0/4
	Control		165.8±20.6	7.48±0.77	33.8±1.6 (32—36)	0/5
28	Inoculated		183.7± 3.8	5.56±0.78[f]	33.3±1.5 (32—35)	3/3
	In-contact		209.8±45.1	6.66±1.56	31.5±1.3 (30 -33)	2/4
	Control		214.6±34.6	7.71±1.08	33.4±2.4 (30—36)	0/5

[a]$10^{6.9}$ $TCID_{50}$ per chick of the Bia strain of CAV as cell-free supernatant fluid from the 10th passage in MDCC-MSBl cells; [b]Died at 8 days of age; [c]Culled as moribund at 14-day-old; [d]Killed at 14-day-old; [e]$p<0.01$ compared with control; [f]$p<0.05$ compared with control; [g]Biological body mass gain; [h]Number positive over number tested

depressed with anaemia. They had significantly lower haematocrit values than the uninoculated control chicks at 14 days of age. These birds also grew poorly: mean biological body mass gain of the inoculated group was significantly lower than that of the control group 21 and 28 days after inoculation. One chick died of yolk-sac rupture with accompanying diffuse peritonitis 8 days post-inoculation, and another chick was killed when moribund at 14 days old. This chick had a haematocrit value of 13%, severely atrophied thymus and bursa of Fabricius, yellowish bone marrow, as well as subcutaneous and intramuscular haemorrhages. Histological lesions in the bone mar-

row, thymus, bursa of Fabricius and spleen were similar to those found in the inoculated chicks of Experiment 1. Surviving inoculated chickens had pale bone marrow and petechial haemorrhages in the skeletal muscles but no other gross lesions when killed at the end of the experiment. Serum antibody to CAV was detected by the indirect IF test in surviving inoculated chicks in samples collected 14 to 28 days after inoculation. Also, CAV was reisolated in MDCC-MSB1 cells from the livers of three out of three chicks examined at 14 and 28 days old.

One-day-old chicks placed in direct contact with inoculated chicks throughout the experiment showed no clinical signs or anaemia. One bird with a haematocrit value of 28% was killed at 14 days of age. No gross lesions were found at necropsy. However, histological examination revealed atrophy of the bone marrow with scattered small regenerative areas consisting of proliferating immature haematopoietic cells, and mild to moderate lymphoid depletion in the thymus and bursa of Fabricius. Two of four surviving chicks had developed antibodies to CAV at 28 days of age, and CAV was isolated in MDCC-MSB1 cells from one of two chicks tested at the end of the experiment.

Uninoculated control chickens of each inoculation experiment showed no clinical or pathological changes, and experienced no mortality during the course of the experiments. All their serum samples collected at 7 to 28 days of age were negative for antibodies to CAV as demonstrated by the indirect IF assay.

Discussion

This study has demonstrated, by both serology and virus isolation, natural CAV infection in young broiler chicken flocks where a sizable proportion of the birds was severely retarded in growth. The clinical signs, postmortem lesions and histopathological changes were similar to those observed in naturally occurring outbreaks of CAV-induced infectious anaemia in young chickens reported by others (Chettle et al., 1989; Goryo et al., 1985; Vielitz and Landgraf, 1988; Yuasa et al., 1983, 1987).

In MDCC-MSB1 cell cultures, a chloroform-resistant virus smaller than 50 nm in diameter, resistant to heating at 70 °C for 30 min and antigenically very closely related to the reference Cux-1 strain of CAV was isolated from the livers of naturally diseased broilers. These properties are consistent with those reported for other CAV isolates (Bülow et al., 1983; Engström, 1988; Goryo et al., 1985, 1987; McNulty et al., 1989*b*, 1990*a*; Yuasa and Imai, 1986; Yuasa et al., 1979), indicating that the isolated virus belongs to the CAV strains, and it has therefore been designated the Bia strain of CAV. Results of cross-indirect immunofluorescence test employing polyclonal chicken antisera strongly suggest that it is of the same serotype as the Cux-1 strain of CAV.

Inoculation of susceptible 1-day-old SPF chicks with the native CAV-positive liver extract from naturally diseased broilers or with the MDCC-MSB1 cell-culture-propagated new Bia strain of CAV caused impaired growth, severe anaemia with hypoplasia or atrophy of the bone marrow, marked atrophy of the lymphoid organs and petechiation throughout the body. These pathological changes strongly resemble those observed by others in SPF chickens after experimental infection at 1-day-old with known strains of CAV (Bülow et al., 1986*a*; Goryo et al., 1985, 1987, 1989; Lucio et al., 1990; McNulty et al., 1990*a*; Yuasa and Imai, 1986; Yuasa et al., 1979).

Chickens experimentally inoculated at 1-day-old with the filtered chloroform-treated, CAV-positive liver extract from naturally diseased broilers did not develop anaemia, even though most of these birds became infected and seroconverted to CAV by 28 days of age. This failure to induce anaemia was undoubtedly due to a low input virus dose used for inoculation, because a considerable proportion of infectious CAV was retained during filtration of the liver extract through consecutively employed filter membranes with decreasing pore sizes, a phenomenon which has previously been observed by others (Engström, 1988; Goryo et al., 1987). McNulty et al. (1990*b*) demonstrated that the ability of CAV to produce anaemia in experimentally inoculated chicks is dose dependent, and a virus dose of at least $10^{3.3}$ $TCID_{50}$ is required to induce anaemia at all. In the present experiment, chicks received $10^{3.07}$ $TCID_{50}$ virus which is less than the above virus dose.

CAV has been reported to spread rapidly among chicks in a group (McNulty et al., 1988, 1990*a*, *b*; Vielitz and Landgraf, 1988; Yuasa et al., 1979, 1980). In this study the virus was also found to spread readily among chickens as evidenced by the presence of virus and serum antibodies in chicks held in direct contact with others inoculated with the field isolate of the virus shortly after hatching. In accordance with previous findings (McNulty et al., 1989*b*, 1990*a*, *b*; Vielitz and Landgraf, 1988; Yuasa et al., 1979, 1980), contact-exposed chicks did not show any clinical signs of the disease. However, we observed atrophy of the bone marrow, with scattered hyperplastic areas of immature haematopoietic cells, and lymphocytic depletion in the thymus and bursa of Fabricius of a killed bird with a haematocrit level of 28% at 14 days of age. These indicate that subclinical pathological changes may also occur in susceptible chicks even after contact exposure. Thus, clarification of this point deserves further studies.

The overall mortality due to natural CAV infection in affected chicken flocks is usually between 10 and 20%, but occasionally it may reach 35% or even 60% in complicated outbreaks (Chettle et al., 1989; Vielitz and Landgraf, 1988; Yuasa et al., 1987). Mortality rates after inoculation of susceptible SPF chicks at day-old ranged from 0 to 90%, depending on the strain and dose of virus used (Bülow et al., 1986*a*; Goryo et al., 1989; Lucio et al., 1990;

McNulty et al., 1990*a*, *b*; Yuasa and Imai, 1986; Yuasa et al., 1979). Experimental studies have shown, however, that the pathogenicity of CAV is considerably enhanced by concurrent infection with either MDV, IBDV or REV, owing to the pronounced immunosuppressive effect of these viruses (Bülow et al., 1983, 1986*a*; Otaki et al., 1987, 1988; Yuasa et al., 1980). Pathogenic effects of adenovirus and reovirus are also increased when CAV is present (Bülow et al., 1986*b*; Engström et al., 1988). Although no antibodies were demonstrated against MDV, IBDV and REV as well as avian adenoviruses and reoviruses in serum samples from either naturally diseased or experimentally inoculated chicks of the present study, it cannot be excluded completely that one or another of these viruses or even many of them might have concomitantly been present, since these viruses, with the exception of REV, are ubiquitous in Hungarian chicken flocks, and thus they may have complicated the naturally occurring pathological syndrome in the field. This could be the reason for the high mortality rate of chicks inoculated at day-old with the native liver extract from spontaneously diseased broilers, because the MDCC-MSB1 cell-culture-propagated field isolate, the Bia strain of CAV caused much lower mortality under similar conditions. It is also conceivable, however, that the pathogenicity of this virus strain might have decreased during serial passages in MDCC-MSB1 cells, as it has been reported for the Cux-1 strain of CAV (Bülow and Fuchs, 1986).

During the inoculation experiments, CAV with the same features as the field isolate was reisolated from a number of inoculated and contact-exposed birds, and antibodies to the reference Cux-1 strain of CAV were also demonstrated in surviving chickens in serum samples collected 14 to 28 days after infection.

The results of the present study fulfil Koch's postulates. Therefore the foregoing experimental evidence allows the conclusion that the naturally occurring disease of young broiler chickens described in this paper was associated with infection by a new isolate of CAV. Physical and chemical, antigenic and pathogenicity characteristics suggest that this virus is similar to CAV strains isolated from chickens in other parts of the world.

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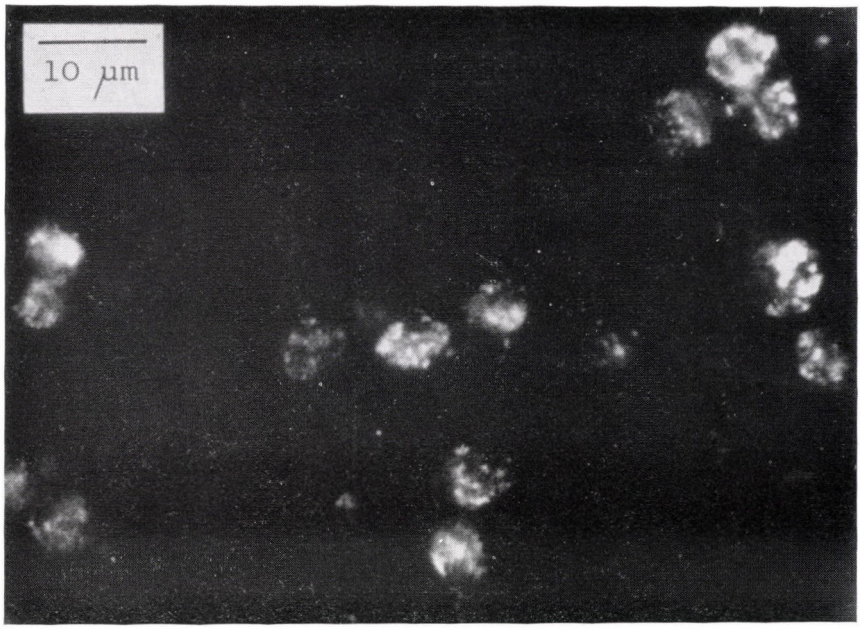

Fig. 1. Granular intranuclear immunofluorescence in MDCC-MSB1 cells infected with chicken anaemia virus isolated from naturally diseased broiler chickens. The cells were reacted with anti-CAV-Cux-1 chicken immune serum and stained with fluorescein-isothiocyanate-labelled anti-chicken IgGMA rabbit immune serum

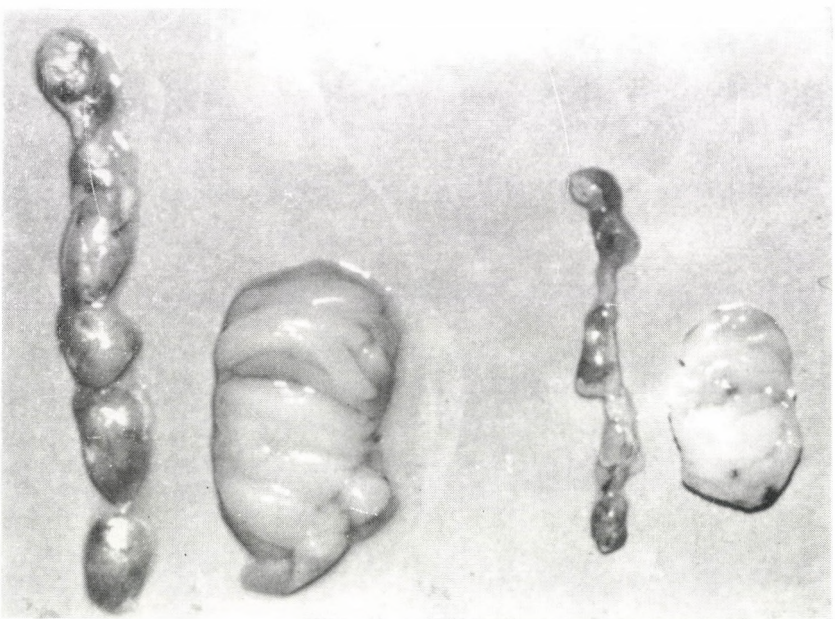

Fig. 2. Thymus and bursa of Fabricius of a 14-day-old uninoculated control chick (left) and of a 14-day-old inoculated chick showing clinical chicken anaemia (right). Both organs of the inoculated bird are severely atrophied, and petechial haemorrhages are present in its bursa

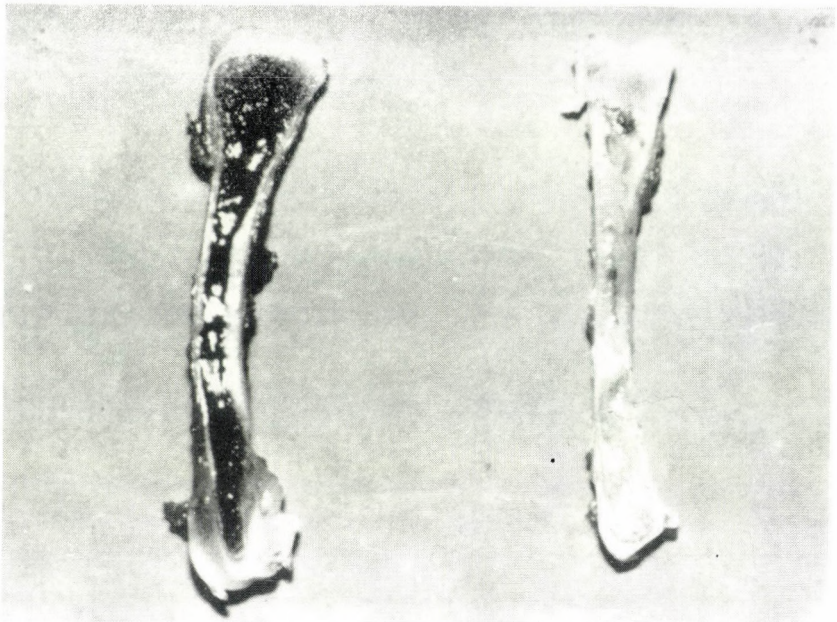

Fig. 3. Longitudinal section of femur from a 14-day-old uninoculated control chick (left) and from a 14-day-old inoculated chick showing clinical chicken anaemia (right). Note discolouration of the bone marrow in the affected bird

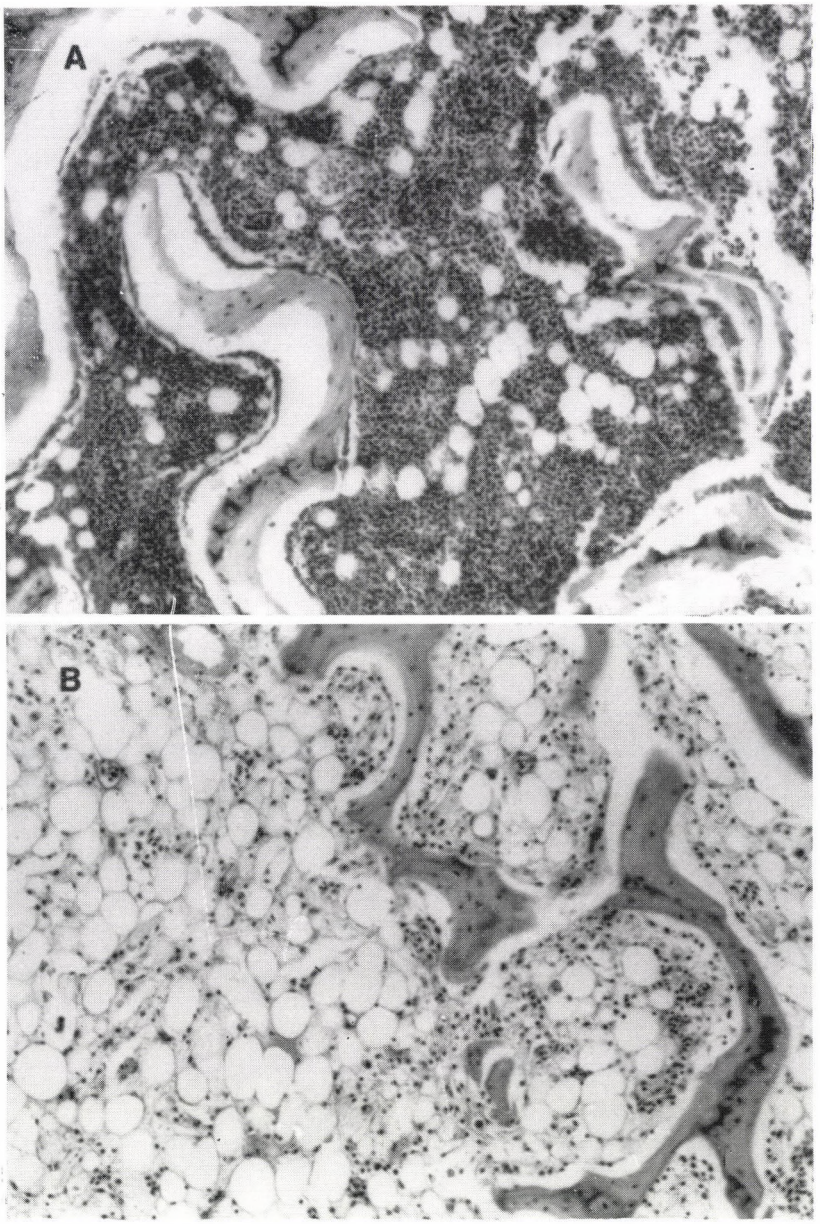

Fig. 4. Sections of femoral bone marrow from 13-day-old chickens. (A) Uninoculated control chick. (B) One-day-old inoculated chick that died at 13 days of age. Atrophy showing almost complete depletion of haematopoietic cells with replacement by fatty tissue. Haematoxylin and eosin, × 206

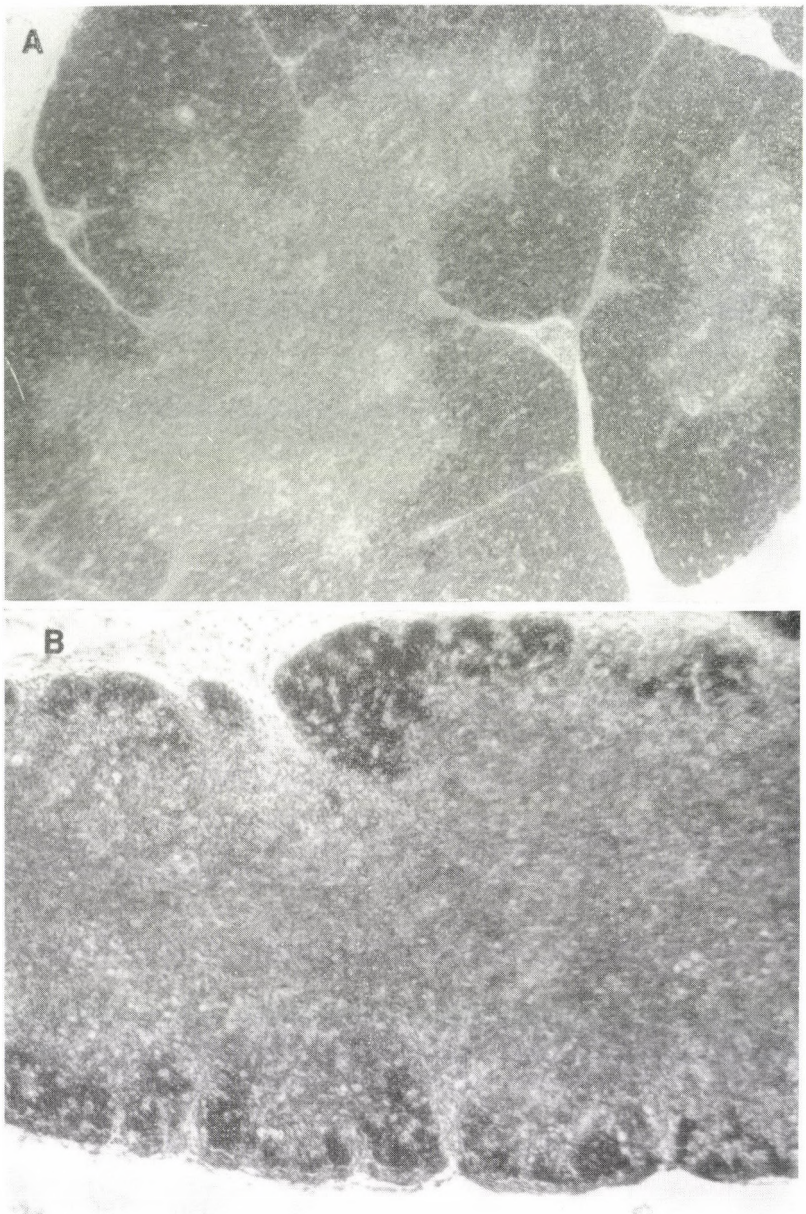

Fig. 5. Sections of thymus from 13-day-old chickens. (A) Uninoculated control chick. Haematoxylin and eosin, × 80. (B) One-day-old inoculated chick that died at 13 days of age. Severe atrophy of the cortex showing generalized depletion of lymphocytes. Haematoxylin and eosin, × 98

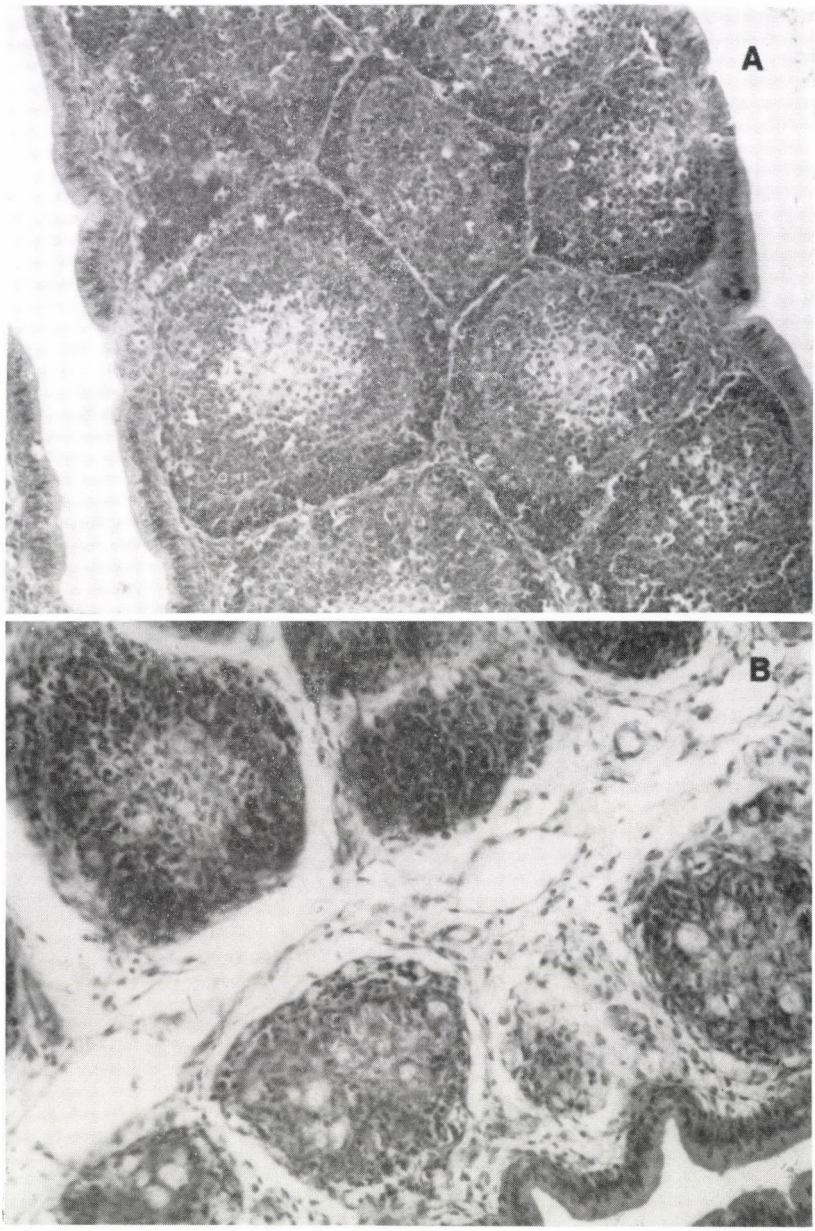

Fig. 6. Sections of bursa of Fabricius from 13-day-old chickens. (A) Uninoculated control chick. Haematoxylin and eosin, × 206. (B) One-day-old inoculated chick that died at 13 days of age. Moderate atrophy with depletion of lymphocytes in the affected follicles. Haematoxylin and eosin, × 260

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STUDY OF BOVINE HERPESVIRUS TYPE 1 STRAINS WITH MONOCLONAL ANTIBODIES

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Fourteen strains of bovine herpesvirus type 1 (BHV-1,IBRV) representing all three groups of BHV-1 (BHV-1.1, BHV-1.2, BHV-1.3) were studied by ELISA using 106 monoclonal antibodies (Mabs) produced against BHV-1. On the basis of the ELISA, the Mabs could be divided inso three groups. The first group (40 Mabs, 38%) reacted with all strains, the second group (43 Mabs, 41%) with the respiratory and genital strains (BHV-1.1 and BHV-1.2) while the third group (23 Mabs, 22%) only with the respiratory strains. Only 5 out of the antibodies neutralized respiratory and genital strains, and none of them neutralized the encephalitogenic strains (1.3).

Three Mabs selected from each of the 3 groups, and the above five neutralizing strains were studied by Western blot. Antibodies of groups 1 and 3, and two neutralizing antibodies bound to a 90k protein (gpIII), whereas members of group 2 and 3 netralizing antibodies reacted with a 74k and a 130k protein (both gpl). The results indicate that reactivity with monoclonal antibodies is as suitable for the classification of BHV-1 strains as is restriction endonuclease (RE) analysis but it cannot distinguish between subgroups within the groups.

Keywords: Bovine herpesvirus type 1, monoclonal antibodies, ELISA, classification

Infectious bovine rhinotracheitis (IBR) is a viral disease causing substantial economic losses in cattle populations worldwide. Its causative agent is IBR-IPV virus which is taxonomically defined as bovine herpesvirus type 1 (BHV-1). The virus has been isolated from different organs and pathological entities and, thus, has been associated with a variety of clinical signs and diseases (pneumonia, inflammation of the upper respiratory tract and nasal mucosa, conjunctivitis, inflammation of the genital organs, abortion, encephalitis).

The marked diversity of pathological entities caused by IBR soon called for a grouping based upon the biological properties of the virus strains. Attempts to group the strains by their morphological properties and replication in cell culture have failed. Polyacrylamide gel electrophoresis of the viral proteins has yielded contradictory results. Initially, the results obtained by different research teams using restriction endonuclease (RE) analysis of the viral DNA were inconsistent. Certain groups (Gregersen et al., 1985; Metzler et al., 1985) reported a correlation between the DNA restriction endonuclease patterns of the virus strains and the clinical signs produced, while others (Misra

et al., 1983; Seal et al., 1985) contested the existence of such a correlation. The first grouping system based upon RE analysis of the viral DNA was proposed by Metzler et al. (1985).

By cleaving the DNA of BHV-1 strains isolated from different pathological entities with restriction endonucleases *Hind*III, *Eco*RI and *Bst*EII, Metzler et al. (1985) could clearly distinguish between BHV-1 strains causing respiratory, genital and encephalitic signs. They designated the respiratory group BHV-1.1, the genital group BHV-1.2 and the encephalitogenic one BHV-1.3. Using restriction endonuclease *Hind*III, they distinguished two subgroups (BHV-1.2a, BHV-1.2b) within the genital group. With *Bst*EII, Magyar (personal communication) found two subgroups (BHV-1.1a, BHV-1.1b) within the group of respiratory strains. Sometimes BHV-1.1 type strains are isolated from cases of encephalitis and BHV-1.2 strains from animals showing respiratory signs. Despite this fact, a correlation between the DNA restriction pattern and the clinical signs produced is demonstrable for the majority of BHV-1 strains.

The aim of this study was to determine whether the above difference can be demonstrated in the antigenic structure of virus strains belonging to different subgroups of BHV-1. As monoclonal antibodies (Mabs) are the most suitable for detecting minimal antigenic differences, in this work we attempted to classify BHV-1 strains on the basis of their antigenic structure using Mabs.

Materials and methods

Virus strains and monoclonal antibodies. In our laboratory, 33 Hungarian BHV-1 isolates and an Australian encephalitogenic strain was studied by RE analysis of the DNA using *Hind*III, *Bst*EII and *Eco*RI enzymes according to Metzler et al. (1985). Four strains belonged to group 1.1a, sixteen to 1.1b, seven to group 1.2a, five to 1.2b, and two to 1.3. Three strains representing the given group or subgroup were selected from each group (only two strains were available in 1.3), and these 14 strains were used for the ELISA. Each of the virus strains was reacted with the 106 Mabs raised against the respiratory strains of BHV-1. Twenty-five Mabs against respiratory strain HB-144 were produced at the Department of Epizootiology of the University of Veterinary Science, Budapest, and 81 clones against a local respiratory isolate were obtained at the Istituto Zooprofilattico Sperimentale (Brescia, Italy).

Viral antigen preparation. Calf testicle cell cultures were prepared in 300 cm^2 cell culture flasks. Upon reaching confluency, the cultures were inoculated with approx. 10^6 PFU of one of the 14 test strains. Forty-eight h after inoculation, upon the development of 80% cytopathic effect (CPE), the contents of the cell culture flasks were frozen and thawed 3–5 times, and cell

debris was removed by centrifugation (6000 g, 20 min). From the supernatant, the viruses were pelleted by ultracentrifugation (Beckman-28 rotor, 100,000 g, 90 min). The virus pellets were resuspended in 1 ml NTE buffer (0.01 M Tris-Hcl, 0.15 M NaCl, 0.01 M EDTA; pH 7.2), layered onto a 30% sucrose cushion made with NTE, and ultracentrifuged as above. The pellet was resuspended in 1 ml NTE buffer.

Production of monoclonal antibodies. The viral antigen prepared as described above was mixed to Freund's adjuvant and used for immunizing Balb/c mice. After subcutaneous inoculation of mice with the virus antigen mixed to Freund's complete adjuvant, immunization was repeated twice at two-week intervals using incomplete adjuvant. Spleen cells from mice having antibodies of at least 1 : 2000 titre in the ELISA were used for fusion. As myeloma cells, SP2 tumour cells were used, at a splenic lymphocyte/myeloma cell ratio 2 : 1. The fusion and hybridoma culture were carried out as described by Kohler and Milstein (1975), using RPMI 1640 (Serva), HAT and HT (Gibco) media and PEG 1500. Hybridoma clones were checked for positivity by ELISA, positive clones were cloned twice by limiting dilution, then ascites fluid was produced by intraperitoneal inoculation of pristane-pretreated mice with the hybridoma cells.

ELISA. The assay was performed similarly to ELISAs developed for IBR virus so far (Payment et al., 1979; Cho and Bohac, 1985). The 14 purified virus strains were bound to 96-well flat-bottomed Greiner cell culture plates (16 h, 4 °C) using 50 μl coating buffer (1.59 g Na_2CO_3 and 2.93 g $NaHCO_3$ in 1 l of distilled water, pH 9.6). Monoclonal antibodies (50 μl) diluted 1 : 10 in washing buffer were added after three cycles of washing in washing buffer (PBS NaCl 8 g/l, KCl 0.2 g/l, KH_2PO_4 0.2 g/l, Na_2HPO_4 1.15 g/l, Tween-20 0.02%, pH 7.2). The plates were incubated at 37 °C for 1 h. After further three cycles of washing, the plates were incubated with a goat anti-mouse immunoglobulin conjugate bound to peroxidase (50 μl per well, 1 : 2500 dilution). After five cycles of washing, the substrate (OPD) was dissolved in substrate buffer (24 ml 0.1 M citric acid, 26 ml 0.2 M Na_2HPO_4, 50 ml distilled water, pH 5.0) to give a concentration of 0.5 mg/ml, then mixed with 30% hydrogen peroxide at a ratio of 1 : 1750. Adding it to the wells at a volume of 50 μl per well, the emerging colour reaction was stopped 10 min later by adding 2 N sulphuric acid (50 μl per well). The absorbance values were read at 492 nm using a Labsystems Multiskan Plus ELISA instrument. The working dilutions of antigen, antibodies and conjugate were determined by previous titration, and the highest dilution still giving positive reaction was used.

Virus neutralization test. Into the wells of 96-well flat-bottomed Greiner cell culture plates we measured ascites fluid containing monoclonal antibody diluted 1 : 10 in serum-free culture medium, and serum-free culture medium containing 100 $TCID_{50}$ virus at a ratio of 1 : 1 (25 μl : 25 μl). The plates were

incubated at 37 °C for 1 h, then 140,000 calf testicle cells in 200 μl were added to each well. The cultures were evaluated for CPE 48 h later.

Western blot. Three Mabs randomly selected from each of the three Mab groups formed on the basis of the ELISA results, and the five neutralizing antibodies were tested by Western blotting. The proteins of the ISCOM vaccine (Merza et al., 1988) containing only the membrane glycoproteins of BHV-1 virus were separated by polyacrylamide gel electrophoresis using a BIO-RAD Mini-Protein II instrument, under reducing conditions, through a 11.1% gel, for 1.5 h. The separated proteins were transferred onto nitrocellulose membrane with an Ancos Semi-Dry Electroblotter instrument (Denmark). The membrane was cut into 3 mm wide strips and kept in a petri dish at –20 °C until used. The strips were saturated by soaking them in 3% BSA solution for 1 h, then were washed (in distilled water for some seconds and in PBS for 2×10 min). The strips were incubated overnight at 4 °C in ascites fluid diluted 1 : 100 with PBS. After washing, the strips were soaked in goat anti-mouse immunoglobulin solution (HUMAN Vaccine Research Institute, Budapest) bound to 1 : 1000 diluted peroxidase at room temperature for 3 h. After a further washing, 0.67 ml 3-amino-9-ethylcarbazol solution dissolved in dimethyl sulfoxide at a concentration of 0.4%, 10 ml 0.1 M sodium acetate (pH 5.2) and 10 μl 30% hydrogen peroxide were used to elicit the colour reaction. Pharmacia's low molecular weight kit was used as molecular marker.

Results

On the basis of results of the ELISAs, the Mabs could be divided into three groups. Group 1 included those 40 Mabs which reacted positively with all virus strains. The 43 Mabs assigned to Group 2 reacted only with the respiratory and genital strains and not with the encephalitogenic isolates. The 23 Mabs included in Group 3 gave a positive reaction only with the respiratory strains (Table 1). The ELISA failed to differentiate among the subgroups (1.1a, 1.1b and 1.2a, 1.2b); it only distinguished the three main groups, i.e. the respiratory, genital and encephalitogenic strains.

Table 1

Reactions of monoclonal antibodies in ELISA, virus neutralization (VN) and Western blotting

Monoclonal antibody	ELISA Respiratory	ELISA Genital	ELISA Encephalitogenic	VN	Western blot
Group 1 (40 Mabs, 38%)	+	+	+	—	gpIII
Group 2 (43 Mabs, 41%)	+	+	—	5	gpI, gpIII
Group 3 (23 Mabs, 22%)	+	—	—	—	gpIII

Only 5 out of the 106 Mabs neutralized the respiratory and genital strains in the virus neutralization test. None of the Mabs neutralized any of the two encephalitogenic strains.

In Western blotting, the tested Mabs reacted with the gpIII glycoprotein (90 k). A certain part of the Group 2 Mabs bound to the gpI glycoprotein (74 k, 130 k).

Discussion

The results of this work indicate that, similarly to DNA restriction endonucleases, Mabs are suitable for grouping BHV-1 viruses, and the results obtained with them show good agreement with those of the RE analyses. In the antigen-antibody reactions (ELISA, virus neutralization, Western blot), the virus strains reacted according to Metzler's grouping based on DNA structure. A clear correlation was demonstrable between the genome and antigenic structure of the viruses.

As RE analysis of the viral DNA is a material-intensive and time-consuming method, Mabs in a simple ELISA are suitable for the classification of BHV-1 strains. If a Mab is selected from each of the three Mab groups, and these three Mabs are used for testing a given isolate by ELISA, the isolate is *respiratory* if it reacts with all three Mabs. *Genital* strains react with two Mabs, while *encephalitogenic* strains with a single Mab only.

RE analysis of the viral DNA has proved to be more sensitive: as restriction enzymes can differentiate, in addition to the three large groups, also subgroups (*Hind*III within the genital and *Bst*EII within the respiratory strains), Mabs can discriminate only between the three main groups and fail to distinguish between subgroups.

Forty-one per cent of the Mabs failed to react with the encephalitogenic strains, and none of the neutralizing antibodies neutralized them.

These substantial dissimilarities in antigenic structure may be related to the fact that in DNA structure encephalitogenic strains (BHV-1.3) differ from respiratory (BHV-1.1) and genital (BHV-1.2) strains more than the latter differ from each other.

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EFFECT OF BULK FEEDS (ALFALFA HAY, CORN SILAGE) ON THE METABOLISM AND LIVER PARAMETERS OF GROWING GEESE

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Rearing experiments were conducted with a total of 90 liver hybrid geese from Babat, divided into three groups of 30 birds each. The effect exerted by all-concentrate feeding (group 1), concentrate feeding supplemented with alfalfa hay (group 2) or with corn silage (group 3) *ad libitum* on the blood glucose level, blood plasma total lipid, total cholesterol and free fatty acid level, and on total lipid content of the liver was studied. In addition, aspartate aminotransferase (AST) activity of the blood plasma and carotene, vitamin A and vitamin E concentration of the blood plasma and the liver were determined. It was found that the blood and liver parameters of goose groups fed different diets changed within the physiological limits typical of the species. Excessive fibre intake resulted in reduced lipid transport within the organism at an unchanged plasma cholesterol level; at the same time, blood glucose level remained unchanged. *Ad libitum* feeding of alfalfa hay and corn silage enhanced carotene and vitamin A transport and carotene storage but did not affect the transport of vitamin E. The results confirm earlier data of the literature that β-carotene and vitamin A together impair vitamin E metabolism.

Key words: Goose, carotene, vitamin A, vitamin E, bulk feed, lipid, carbohydrate, cholesterol

Several studies have been published on the effect of crude fibre on carbohydrate, lipid and vitamin metabolism. In their comprehensive work, Wolever and Jenkins (1986) reported that in vertebrates the increase in dietary fibre intake is usually accompanied by a decline in the blood glucose level, adding that this effect depended not only on the quantity but also on the quality of the fibre. Most authors agree that crude fibre affects the absorption of fatty acids and cholesterol in poultry (Fisher and Griminger, 1976). Different types of fibre may have dissimilar effects. The liver of chickens (Akiba and Matsumoto, 1980) and laying hens (McNaughton, 1978) fed high levels of alfalfa meal contained lower levels of lipid and cholesterol. If the fibre level of laying hen diets was increased by adding sunflower seed meal, hepatic cholesterol concentration was found to rise (McNaughton, 1978). In our previous paper (Vetési et al., 1990), we reported on the effect exerted by the *ad libitum* feeding of greens as a supplement to concentrate on the metabolism of growing geese. The aim of this work was to determine whether the supplementation of concentrate with *ad libitum* fed alfalfa hay or corn silage rich in

fibre, aimed at reducing the costs of rearing, induces changes in the carbohydrate, lipid and vitamin metabolism of geese, in the integrity of liver cells, and in the lipid and vitamin metabolism of the liver.

Materials and methods

Liver hybrid geese from the Babat goose farm were used in the experiments (males and females in the same number). The geese were reared from day-old to 10-week-old age. The rearing conditions corresponded to the technological specifications commonly used in Hungary. All groups were kept, fed and watered in the same way. In addition to the concentrate mix described in Table 1, 20 g chopped alfalfa hay was fed to each bird every day to avoid feather and litter picking (group 1, control group). In addition to the concentrate mix, the experimental groups received alfalfa hay *ad libitum* (group 2; alfalfa hay fed group) or corn silage (group 3, corn silage fed group). Each group consisted of 30 birds. The quantity of concentrate and bulk feed consumed was measured daily. Daily average crude fibre intake could be calculated accurately from the feed consumption data and from the dietary fibre content checked regularly.

For laboratory tests aimed at determining the metabolic status and condition of the liver, blood and liver samples were taken from 10 eight-week-old geese in each group.

The activity of aspartate aminotransferase (AST; E.C.2.6.1.1) indicative of the physiological status of the liver was determined in the blood plasma by the colorimetric method described by Reitmann and Frankel (1957).

Of the parameters of carbohydrate metabolism, glucose concentration of the blood plasma was determined by Trinder's method (1969).

Of the parameters of lipid metabolism, total lipid, total cholesterol and free fatty acid concentration of the blood plasma and total lipid content of the liver were monitored. Total lipid content of the blood plasma was measured by the sulfophospho-vanillic acid reaction (Christie, 1982). Total cholesterol concentration was determined by Watson's (1960) enzymatic procedure, while the concentration of free fatty acids was measured by the colorimetric method of Duncombe (1964).

The carotene, vitamin A and vitamin E concentration of blood plasma and liver samples taken from 8-week-old geese was determined. Total carotene and vitamin A content of the blood plasma was measured by spectrophotometry according to Bárdos (1988). Total carotene and vitamin A content of the liver samples was also determined by spectrophotometry after saponification and extraction with petroleum ether (Bárdos, 1988; Mézes, 1979). The vitamin E content of the blood plasma was determined by Bieri's (1964)

Table 1

Composition and nutrient content of basal diet

Ingredients and analyses	Percentage
Ground corn	60.20
Sunflower meal (45%)	20.50
Alfalfa meal	8.00
Torula yeast	1.00
Fat powder (50%)	3.00
Biometin (20% methionine)	1.50
Biolizin (20% lysine)	1.80
Vitamin and trace mineral premix	4.00
Total	100.00
Calculated analyses	
AME_n(MJ/kg)	11.70
Protein	16.00
Ether extract	3.50
Crude fiber	5.30
Methionine + cystine	0.84
Methionine	0.60
Lysine	0.94
Calcium	1.20
Phosphorus (total)	0.60
Crude protein/AME_n (g/MJ)	13.60
Lysine/AME_n (g/MJ)	0.80
Methionine + cystine/AME_n (g/MJ)	0.71

[1] Provided per kg of premix: Vitamin A, 225,000 IU; vitamin D_3 66,000 IU; vitamin E, 250 IU; vitamin K, 37.50 mg; vitamin B_2, 120 mg; Ca-d-pantothenate, 300 mg; vitamin B_{12}, 0.25 mg; niacin, 875 mg; choline-chloride, 7500 mg; EMQ, 2062.50 mg; zinc, 1450 mg; copper, 100 mg; iron, 500 mg; iodine, 12.50 mg; manganese, 1450 mg; selenium, 6.12 mg; phosphorus, 5.52%; calcium, 22.85%; salt, 7.50%

spectrophotometric method. Vitamin E content of the liver samples was measured according to Haacker (1977).

Statistical analysis of the results was performed by Student's two-sample *t*-test.

Results

The feed consumption and crude fibre intake of the geese are shown in Table 2. The geese of group 2 (alfalfa hay fed group) consumed three times as much crude fibre as the control birds, while the birds in group 3 (corn silage fed group) took up 60% more crude fibre than the controls.

Table 3 contains the values of blood parameters indicative of energy metabolism and of parameters reflecting liver condition. The results show that the feeding of alfalfa hay or corn silage failed to induce substantial changes in blood plasma glucose concentration.

Table 2

Average daily intake of feed and crude fibre (g/day)

	Group		
	1	2	3
Average daily concentrate intake	191	187	186
Average daily alfalfa hay intake	10	142	—
Average daily corn silage intake	—	—	133
Average daily crude fibre intake	10.10	30.60	16.60

Table 3

Effect of dietary fibre intake on blood parameters indicative of carbohydrate and lipid metabolism and physiological status of the liver (mean ± S.D.)

Parameter/group	1	2	3
Number of samples	10	10	10
Plasma glucose (mmol/L)	4.02 ± 1.54	5.50 ± 0.41	4.22 ± 0.73
Plasma lipid (g/L)	5.93 ± 0.70	4.51** ± 0.56	5.11** ± 0.55
Plasma FFA (mmol/L)	0.30 ± 0.05	0.25* ± 0.03	0.24 ± 0.06
Plasma cholesterol (mmol/L)	4.28 ± 0.12	4.50** ± 0.11	4.28 ± 0.66
Plasma AST activity (U/L)	14.00 ± 3.10	12.50 ± 3.51	12.50 ± 2.26

Levels of significance: * = $P < 0.05$ 1 vs 2; 1 vs 3
** = $P < 0.01$ 1 vs 2; 1 vs 3

Total lipid concentration of the blood plasma and liver was in the physiological range in all three groups. It was remarkable that lipid concentration was lower in the groups given bulk feeds *ad libitum* than in the control group. The concentration of free fatty acids indicative of lipid mobilization was also in the physiological range. Free fatty acid concentration was lower in both experimental groups than in the control group (average value: 0.3 mmol/l); however, only in the case of group 3 was that difference statistically significant.

The blood cholesterol values measured can be considered physiological (4.28–4.50 mmol/l), and the differences found among the three groups are biologically negligible, though the blood cholesterol concentration of the alfalfa hay fed group (group 2) significantly exceeded that of the control.

Elevated blood plasma AST activity indicative of liver injury could not be observed in any of the groups. The minor differences measured between the mean values (12.5–14.0 U/L) have no practical importance.

The blood of geese fed alfalfa hay and corn silage contained higher levels of carotene and vitamin A, and carotene storage was more pronounced, than in the controls. At the same time, the storage and transport of vitamin E were practically unchanged (Table 4). The transformation of carotene into vitamin A seemed to be more efficient in the control group than in the experimental groups fed a bulk feed (alfalfa hay or corn silage) *ad libitum*.

Table 4

Carotene, vitamin A and vitamin E content of plasma and liver (mean ± S.D.)

Parameter/group	Plasma	Liver
Carotene (mg/L, mg/g)		
1	0.89 ±0.18	0.37 ±0.03
2	1.40*** ±0.17	0.74** ±0.17
3	1.35** ±0.27	0.69* ±0.27
Vitamin A (IU/L, IU/g)		
1	2630.81 ±153.37	40.56±21.32
2	3202.21** ±302.94	43.05± 6.36
3	2952.22** ± 73.77	44.30±19.98
Vitamin E (mg/L, μg/g)		
1	2.20±0.20	6.74±0.39
2	2.28±0.24	7.02±0.62
3	2.38±0.25	7.25±0.51

Levels of significance: * = $P < 0.05$ 1 vs 2; 1 vs 3
** = $P < 0.01$ 1 vs 2; 1 vs 3
*** = $P < 0.001$ 1 vs 2; 1 vs 3

Discussion

The glucose concentrations measured in the blood plasma (4.02–5.50 mmol/l) can be considered physiological (Mészáros, 1976; Szép et al., 1976). Story and Kritchevsky (1976) found that different types of fibre have dissimilar effects on the blood glucose level, and that the effect exerted by crude fibre is influenced also by the level and quality of dietary protein and fat. At the feeding regimen used in our case, high dietary fibre intake — derived either from alfalfa hay or from corn silage feeding — caused no significant changes in the birds' blood glucose level.

From data of the literature it appears that the effect of fibre on lipid metabolism depends not only on the absolute intake of fibre or the relative amounts of the different fibre fractions, but also on the protein and lipid supply status (Story and Kritchevsky, 1976). Total lipid content of the blood plasma and liver samples tested was within the physiological limits (Hunsaker and Hunt, 1964); however, in the control geese it was significantly higher than in those given alfalfa hay or corn silage *ad libitum* in addition to the concentrate fed. However, even the values measured in the control group are not suggestive of fatty degeneration of the liver.

Cholesterol concentration of the blood plasma was in the physiological range in all groups (Karsai, 1974). Earlier findings obtained in mammals suggest that total lipid and total cholesterol concentration are closely correlated (Karsai, 1974). In this experiment conducted with geese, however, we found

no such correlation. Our data do not support observations according to which saponins taken up with the feed (in our case with alfalfa) would decrease the plasma cholesterol level (Clary et al., 1960; Newmann et al., 1958). Namely, the blood cholesterol level of geese fed also alfalfa (group 2) did not change as compared to the control group.

The level of free fatty acids (FFA), which reflects the degree of lipid mobilization, did not indicate enhanced and pathological lipid mobilization. On the contrary, high fibre intake resulted in lower FFA concentrations.

Blood plasma AST activity, which indicates the physiological status of the liver, was in the normal range typical of the species throughout (Bokori and Karsai, 1969; Horváth and Bokori, 1971), indicating that high fibre feeding did not cause a liver injury detectable by enzyme diagnostic tests. In chickens fed diets supplemented with high-fibre alfalfa meal, pure cellulose or rice husk, Akiba and Matsumoto (1980) observed a decrease in blood plasma AST activity. Similar observations could not be made in this case.

On the basis of blood plasma and hepatic carotene concentration and the vitamin A content of the liver, carotene and vitamin A transport and carotene storage were much more pronounced in goose groups fed alfalfa hay or corn silage than in the geese fed concentrate only. The carotene — vitamin A transformation was, however, more efficient in the control geese, as shown by blood plasma and hepatic carotene and vitamin A concentrations as well as their ratios. This can be attributed partly to the fact that the concentrate mix fed contained sufficient carotene and vitamin A to meet the requirements of geese. Namely, it is known that — as with other nutrients — the relative quantity of absorbed carotene decreases in the case of excess consumption (Shaeffer et al., 1988).

In goose fed bulk feeds (groups 2 and 3), vitamin E content of the blood plasma and liver did not change. This observation supports data of the literature according to which the high level of β-carotene (Bendich and Shapiro, 1986), but even more of vitamin A (Combs, 1976; Frigg and Broz, 1984; Blakely et al., 1990) causes the blood plasma vitamin E level to decrease, and that β-carotene and vitamin A exert a synergistic effect on the metabolism of vitamin E. Presumably, the interaction among carotene, vitamin A and vitamin E can be partly attributed to the fact that these substances are transported in the blood bound to the lipoprotein fractions of the blood plasma and, thus, a certain "competition" develops among them.

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DESCRIPTION OF *DOGIELIUS MOLNARI* N. SP. (MONOGENEA: DACTYLOGYRIDAE) FROM THE GILLS OF AN IRANIAN FRESHWATER FISH, *CYPRINION MACROSTOMUM* (HECKEL)

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The occurrence of a new *Dogielius* species is reported from the gills of *Cyprinion macrostomum (Cyprinidae)*, an endemic fish of the River Tigris water system. The species is described as *Dogielius molnari* n. sp.

Key words: Monogenea, *Dogielius*, new species, Iran

The freshwater fish fauna of south-western Iran belongs to the Mesopotamian fauna region, an intermediate zoogeographical region between the Palaearctic and Indian great regions. The region, with its rivers belonging to the Tigris and Euphrates water system, is mostly populated by endemic fish species (Berg, 1949). Dactylogyrids, the majority of which are strictly host-specific parasites, have been reported to be represented by several unknown species on endemic fishes.

The first monogeneans from Iranian fishes were described by Bychowsky (1949) who reported the occurrence of three *Dactylogyrus* and one *Ancyrocephalus* species from River Karkheh. Other monogeneans have been found by Jalali and Molnár (1990), Molnár and Jalali (1992), and Gussev et al. (1993). Among them, two new *Dogielius* species have been described, *D. mokhayeri* from *Aspius vorax* and *D. persicus* from *Carassobarbus luteus*, *Barbus sharpeyi* and *B. grypus*.

In this paper, *Dogielius molnari* sp. n., a monogenean occurring on the gill filaments of the cyprinid fish *Cyprinion macrostomum* in the River Dez (Persian Gulf, River Tigris Water System), is described and depicted.

Materials and methods

Fish specimens were collected from River Dez (a tributary of River Kharoon, Persian Gulf Water System). Parasites were collected under a stereomicroscope at 40- to 100-fold magnification. Monogeneans were picked off,

with a pipette, from gill scrapings alive, placed under a coverslip and fixed in ammonium picrate solution. Measurements were taken and drawings made using a light microscope fitted up with a drawing equipment. The measurements were taken and the terminology was used as recommended by Gussev (1983). All measurements are given in μm.

Results

Three *Dogielius* specimens were found on the gills of four one-year-old *Cyprinion macrostomum*. They belonged to a species new for the science, the description of which is given below:

Dogielius molnari sp. n. (Fig. 1)

Host: *Cyprinion macrostomum* (Heckel)
Locality: River Dez
Specimens studied: 3
Type material: Holotype deposited in the monogenean collection of the Zoological Department, Natural History Museum, Budapest, Hungary

Description:
Small worms with two pairs of eyes. Body length 468 (450–504), width 132 (126–144). Marginal hooks with a well-projected heel of blade 20.2 (18.2–22.5).

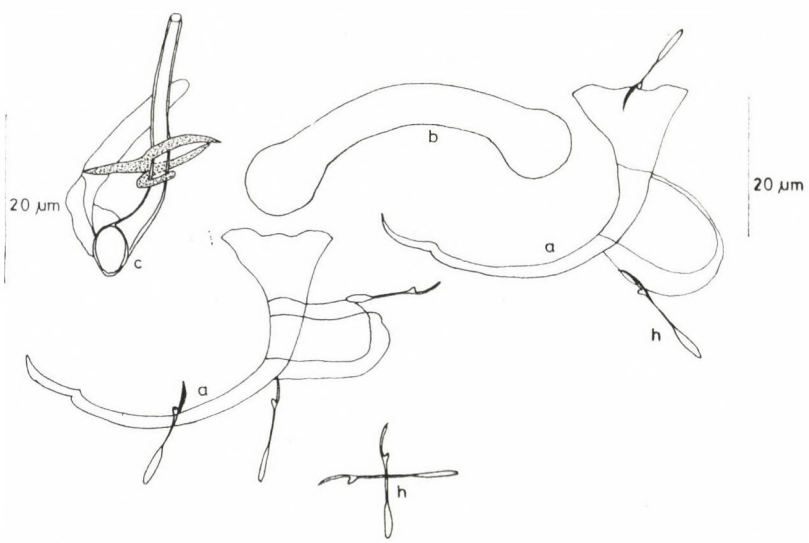

Fig. 1. Schematic illustration of the sclerotized elements of *Dogielius molnari* n. sp. (a) anchors; (b) bar; (c) copulatory organ; (h) marginal hooks

Anchors with less developed outer and inner roots. Points of the anchors open with well-emphasized blades. Ventroapical length of the anchors 48.2 (46.5–51), dorsoapical length of the anchors 31.5 (28.2–33.3). Dorsal root 6.8 (6.1–7.1), ventral root 5.8 (5.5–5.9). The blade of the point 8.5 (8.4–8.6). Connective bar is slightly bent, its lateral ends enlarged. Width of the bar 51 (46.5–59.8), length at the ends 10 (8.3–11.6) in the middle 4.7 (3.8–5.0). Copulatory organ is composed of an elongated tube, with a basal part having an elongated process and a pincers-like accessory part. Length of the copulatory organ 34.1 (31.5–35.7).

Discussion

The genus *Dogielius* was first described by Bychowsky (1936) who reported the occurrence of *D. forceps* from the Central Asiatic region. It was also Bychowsky (1957) who described *D. planus* from the River Amu-Darya. Later on, several species of *Dogielius* were found in Africa (Paperna, 1969, 1973; Guegan et al., 1988; Guegan and Lambert, 1991). On the basis of Jain's data, Gussev (1973–1974) described a *Dogielius* species from the Indian region. From Iran two *Dogielius* species, *D. mokhayeri* and *D. persicus* have been described in the framework of the present study series (Jalali and Molnár, 1990; Molnár and Jalali, 1992). In the latter two papers a regrettable error was committed and the genus name "*Dogielius*" was erroneously written as "*Dogelius*" throughout.

From the results obtained the conclusion can be drawn that *Dogielius* spp. are more widespread species than it was previously believed. Thus, the occurrence of several new species can be expected in fishes in every region of the world except on the American and the European continent.

The species described in this paper was named after dr. Kálmán Molnár, a well-known Hungarian fish parasitologist.

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EFFECT OF ANTHELMINTICS ON PHOSPHATASES IN *ASCARIDIA GALLI*

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In vitro addition of the drugs tetramisole (TMS) and levamisole (LMS) caused an inhibition of the specific activities of acid phosphatase and Mg^{++}-dependent adenosine triphosphatase. The inhibition was non-competitive in nature. No significant inhibition was caused by TMS in the activity of glucose-6-phosphatase, but LMS inhibited the enzyme in a non-competitive manner. The activity of alkaline phosphatase was, however, increased in the presence of both TMS and LMS.

Key words: *Ascaridia galli*, anthelmintics, phosphatases

Phosphatases are known to play a variety of important roles at the transporting surfaces (Lumsden, 1975; Pappas and Read, 1975), in the extracellular digestion and phosphorylation of nutrients transported, secreted and excreted (Maki and Yanagisawa, 1980). Due to their presence at the absorptive surfaces, phosphatases react with the substances in the external milieu (Pappas and Read, 1975). Anthelmintics may alter the enzymes and modify the normal metabolism of the absorptive surfaces during their absorption. Tetramisole (TMS) and levamisole (LMS) are broad-spectrum anthelmintics effective against the common worm infestations of birds, cattle and humans. LMS has been reported to be more active than TMS (Kates et al., 1971), but both have a dual inhibitory action on nematodes by exerting a direct stimulatory effect on muscular contraction (paralytic) as well as an inhibitory effect upon the fumarate reductase system (Bossche, 1972). In this work, the effect of anthelmintics on phosphatases was studied at different substrate concentrations in adult *Ascaridia galli*, in order to get a better insight into the complex enzyme-substrate interaction and the mechanism of inhibition of the enzyme by the drugs.

Materials and methods

Adult specimens of *Ascaridia galli* were collected from the intestine of fowl procured from local poultry farms. The worms were thoroughly washed in saline (0.9% NaCl) to remove debris and adhering materials.

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A 10% homogenate of the worms in their respective buffers or 0.25 M sucrose was centrifuged at 2,000 g for 30 min at 4 °C. The supernatant served as the source of enzyme activity.

Acid (ACP) and alkaline phosphatases (ALP) were estimated by the method of Natelson (1963). Mg^{++}-dependent adenosine triphosphatase (Mg^{++}-ATPase) and glucose-6-phosphatase (G-6-Pase) were estimated by the method of Kieley (1972) and Swanson (1950), respectively.

The effect of TMS and LMS on parasitic enzymes and their Michaelis-Menten kinetic parameters were studied by adding the drugs at a concentration of 0.04 mM (final concentration) to the standard incubation system. This concentration was determined on the basis of initial laboratory experiments where it was found that the worms were completely paralysed. Complete cessation of motility was found with TMS after 45 min and with LMS after 30 min at 37 °C. V_{max} (maximum of apparent initial enzyme velocity) and K_m (substrate affinity constant) of the helminth enzymes were determined by assaying the enzymes at different substrate concentration (Lineweaver and Burk, 1934). Similarly, V_{max} and K_m of the enzymes were determined after *in vitro* addition of TMS and LMS, at the dose level of 0.04 mM of each.

Differences between the mean $\pm$ SD values for worms in different groups were compared by Student's *t*-test. Statistical differences were considered significant when P was 0.05 or less.

Results

The results of enzymatic analysis showing specific activity, percentage inhibition and drug-induced changes in V_{max} and K_m are shown in Tables 1 and 2. *In vitro* addition of the drugs TMS and LMS inhibited enzyme activity in *A. galli*. Varying degrees of inhibition were observed with the drugs: in-

Table 1

Effects of *in vitro* addition of tetramisole and levamisole on the phosphatase enzymes of *Ascaridia galli*

Enzyme (μmol/100 mg protein/min)	Control	TMS	% change	LMS	% change
Acid phosphatase	33.0±2.0	21.±2.5***	36.36	15.0±1.8***	54.54
Alkaline phosphatase	11.9±1.5	15.0±2.0*	+20.66	15.0±2.0*	+20.66
Glucose-6-phosphatase	35.00 ±2.0	32.50±3.0^{NS}	8.57	27.98±2.0***	22.85
Mg^{++}-dependent adenosine triphosphatase	240.00±10.0	150.00±15.0***	37.5	115.00±10.0***	52.08

The results are expressed as mean ± SD of 3–4 observations
*P < 0.05; **P < 0.01; ***P < 0.001; ^{NS}not significant

Table 2

Effects of *in vitro* addition of tetramisole and levamisole on V_{max} and K_m of the phosphatases enzymes of *Ascaridia galli*

Enzyme	V_{max} (μmole/100 mg protein/min)			K_m (μM)		
	Control	TMS	LMS	Control	TMS	LMS
Acid phosphatase	71.42	41.66	31.25	1.11×10^3	1.11×10^3	1.11×10^3
Alkaline phosphatase	20.00	33.33	33.33	0.08×10^3	1.08×10^3	1.08×10^3
Glucose-6-phosphatase	71.42	58.82	50.00	0.08×10^6	0.08×10^6	0.08×10^6
Mg^{++}-dependent adenosine triphosphatase	500	333	250	5.80×10^3	5.80×10^3	5.80×10^3

V_{max} = maximum of apparent initial enzyme velocity; K_m = substrate affinity constant

hibition of the enzymes was more marked in the presence of LMS as compared to TMS.

ACP activity was reduced to 36.3% and 54.5% by TMS and LMS, respectively. K_m was not changed but V_{max} was lowered, hence showing a non-compe-

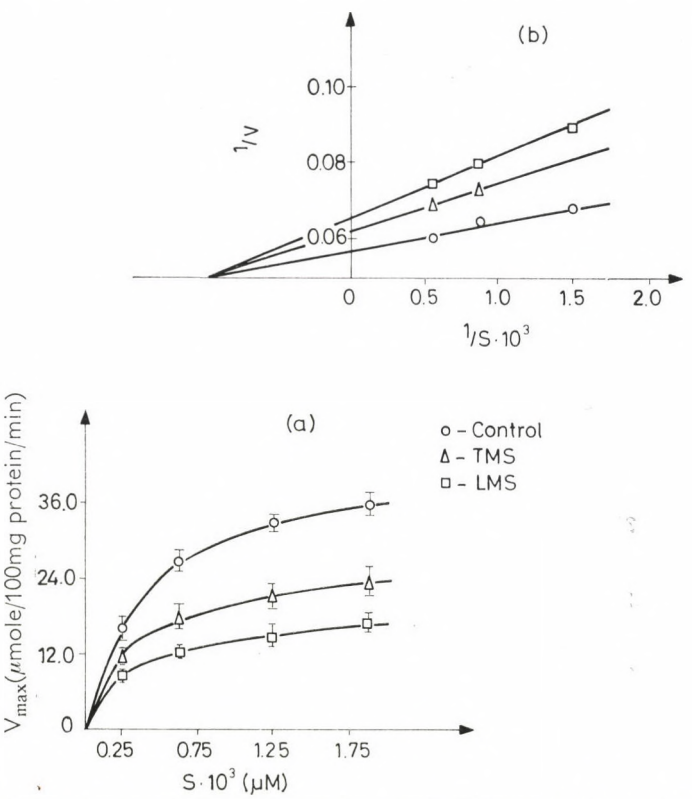

Fig. 1. (a) Effect of tetramisole (TMS) and levamisole (LMS) on acid phosphatase (ACP) in *Ascaridia galli.* (b) V_{max} and K_m of ACP in the presence of TMS and LMS in *A. galli*

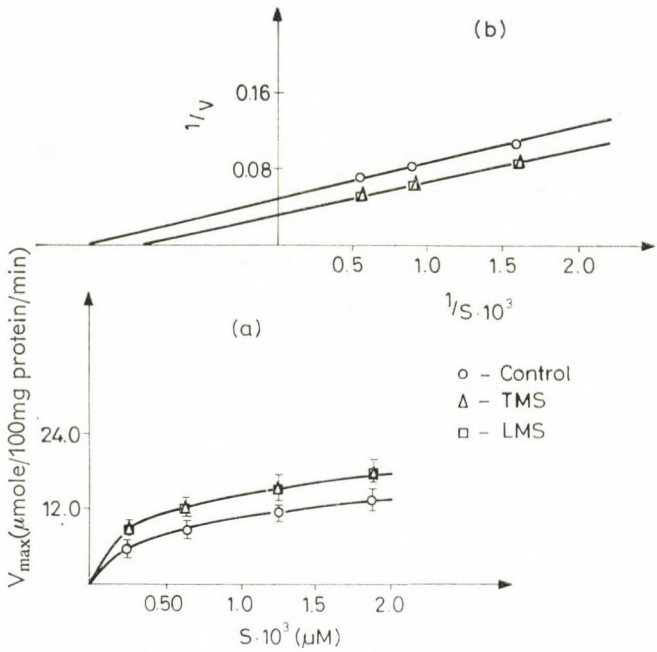

Fig. 2. (a) Effect of TMS and LMS on alkaline phosphatase (ALP) in *A. galli.* (b) V_{max} and K_m of ALP in the presence of TMS and LMS in *A. galli*

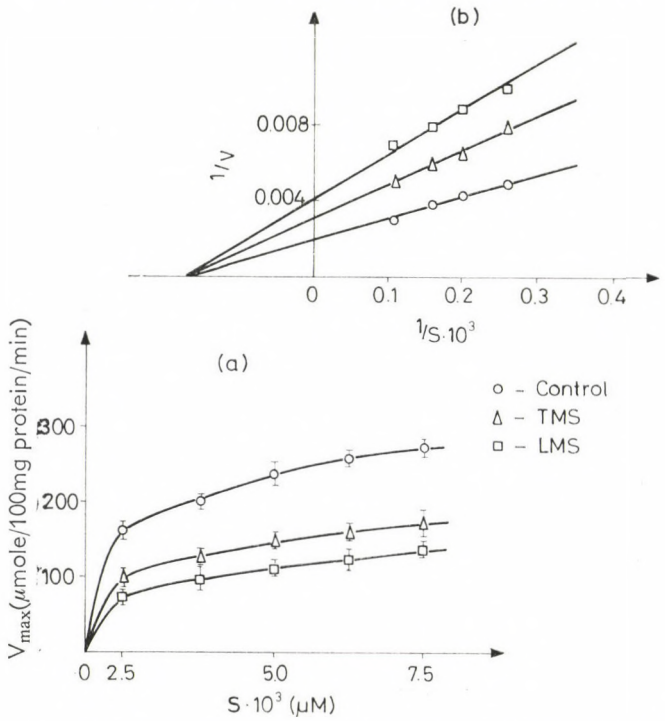

Fig. 3. (a) Effect of TMS and LMS on Mg^{++}-dependent adenosine triphosphatase (ATPase) in *A. galli.* (b) V_{max} and K_m of Mg^{++}-dependent ATPase in the presence of TMS and LMS in *A. galli*

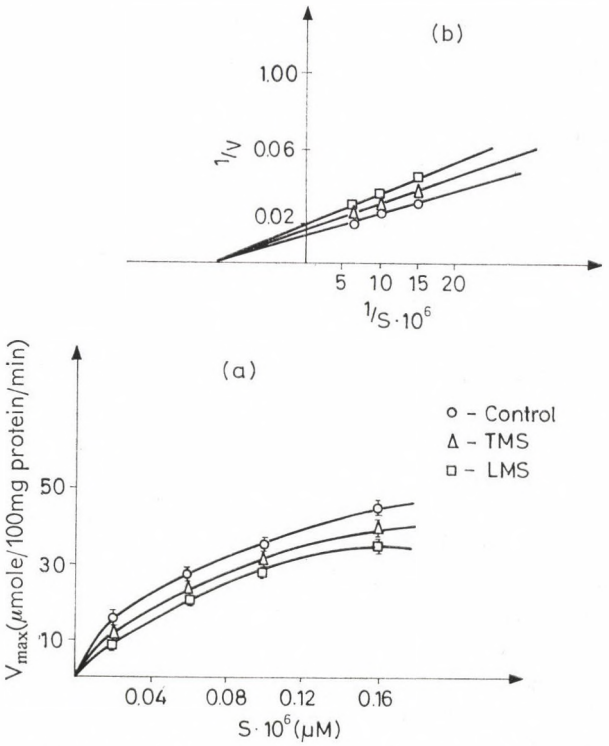

Fig. 4. (a) Effect of TMS and LMS on glucose-6-phosphatase (G-6-Pase) in *A. galli.* (b) V_{max} and K_m of G-6-Pase in the presence of TMS and LMS in *A. galli*

titive inhibition by the drugs (Fig. 1). K_m was elevated in both cases. V_{max} also showed an increase (Fig. 2). ATPase was reduced to 37.5% and 52.1% by TMS and LMS, respectively, showing non-competitive behaviour (V_{max} was lowered but K_m remained unaffected; Fig. 3). TMS caused no significant inhibition in the activity of G-6-Pase but LMS inhibited it up to 22.8% K_m showed no change but V_{max} was lowered (non-competitive behaviour; Fig. 4).

Discussion

In parasitic helminths, phosphatases are believed to be involved directly in the absorption of nutrients. It has been suggested that their presence may be indicative of active transport (Lumsden, 1975). Using histochemical techniques, strong ACP activity was demonstrated in the intestine of a variety of nematodes such as *A. suum* (Borgers and De Nollin, 1975) and *A. galli* (Parshad and Guraya, 1978). These authors suggested that high ACP activity in the intestine of these nematodes might be related to extracellular digestion, absorption and other related functions. ACP activity has also been detected

in the cuticle and hypodermis of *Litomosoides carinii*, *Brugia pahangi* (Maki and Yanagisawa, 1980) and *A. galli* (Parshad and Guraya, 1978), indicating the importance of the body wall as the site of nutrient absorption. Chakrobarty et al. (1976) showed that TMS inhibited ACP activity in *A. galli*. The inhibition of enzyme activity by TMS and LMS as found in this study suggests that the absorption and intracellular digestion of the drug may involve lysosomes (Colan, 1971). A decrease in activity is probably due to its leakage into the medium as a result of the disruption of absorptive surfaces (Borgers and De Nollin, 1975; Hart et al., 1977). The non-competitive nature of enzyme inhibition induced by these drugs (K_m was not changed but V_{max} was lowered) also suggests that substrate binding to the catalytic site of the enzyme was not affected.

In the present study, ALP activity was detected in *A. galli* but was quite low as compared to the activity of ACP. This is consistent with the findings obtained for *Haemonchus contortus* (Kaur and Sood, 1982). Hence, acid phosphatase is the major enzyme involved in the hydrolysis of phosphate esters in gastrointestinal nematodes. The activity of ALP increased upon addition of the drugs TMS and LMS in *A. galli*. Both K_m and V_{max} were affected, indicating that although the substrate affinity of the enzyme was affected adversely, the number of effective enzyme molecules available was quite high. Rafoxanide and TMS treatment in *H. contortus* also increased ALP activity (Kaur and Sood, 1982). Increased ALP activity may indicate certain alterations in the transport processes.

Mg^{++}-dependent ATPase is widely distributed in helminths. Kaur and Sood (1982) demonstrated ATPase activity in *H. contortus* ATPase is known to be related to energy metabolism, active transport and lipid synthesis (Lehninger, 1975). Consequently, any effect on ATPase may result in the alteration of these metabolic processes. The activity of this phosphatase undergoes a reduction in *A. galli* upon addition of both TMS and LMS (LMS causes a more marked reduction). V_{max} is lowered but K_m remains unchanged, showing that the inhibition is of non-competitive nature. ATPase activity has been reported to be reduced to some extent by the drugs rafoxanide and TMS in *H. contortus* (Kaur and Sood, 1982).

G-6-Pase activity showed no significant change in *A. galli* after the addition of TMS while it underwent a significant decrease in the presence of LMS. In *H. contortus*, TMS caused a negligible change in G-6-Pase activity but rafoxanide produced an elevation in the activity of that enzyme (Kaur and Sood, 1982). G-6-Pase presumably has a role in digestion and in absorption of nutrients. No direct evidence exists, however, to support that phosphohydrolases present at the absorptive surfaces furnish any kinetic advantage in the absorption of products formed by the hydrolysis of glucose-6-phosphate and glycerol phosphate (Parshad and Guraya, 1978).

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EFFECT OF ACUTE SALINOMYCIN-TIAMULIN TOXICITY ON THE LIPID PEROXIDE AND ANTIOXIDANT STATUS OF BROILER CHICKEN

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The combined effect, if any, of salinomycin poisoning and salinomycin–tiamulin interaction on lipid-peroxidative processes and the antioxidative defence system of the liver was studied in domestic fowl. Male broilers (28-day-old), reared on a diet containing 60 mg/kg salinomycin, were treated intraoesophageally with salinomycin (140 mg/kg body mass) or tiamulin (50 mg/kg body mass). Malondialdehyde, reduced glutathione and cytochrome P-450 concentrations as well as glutathione peroxidase and catalase activities of the liver were determined. Liver malondialdehyde concentration rose in the salinomycin-treated group while the amount of cytochrome P-450 increased in both groups treated. Glutathione concentration and glutathione peroxidase activity of the liver decreased rapidly but hepatic catalase activity increased in both groups after the treatment. Manifestation of the effect exerted by salinomycin and salinomycin-tiamulin on lipid-peroxidative processes nearly coincided with the onset of clinical signs and preceded the increase of hepatic cytochrome P-450 concentration. According to the results, the background of the previously reported incompatibility between salinomycin and tiamulin is the synergistic effect exerted on the antioxidant (glutathione) system.

Key words: Salinomycin, tiamulin, toxicity, broiler chicken, lipid peroxidation, glutathione

One of the most important drug incompatibilities currently known in the veterinary practice is the interaction between some ionophore antibiotics (e.g. monensin, salinomycin, narasin) and tiamulin. The clinical signs and pathological changes of toxicity caused by such drug incompatibilities have been reported by several authors from poultry (Horrox, 1980; Weisman et al., 1980) and swine (Miller et al., 1986; Romváry and Fisi, 1988; Wanner, 1984).

The mechanism of this toxic interaction is not known yet. In isolated rat liver preparations, tiamulin was found to reduce the degradation and elimination of monensin (Meingassner et al., 1979). The authors concluded that co-administration of these drugs causes an overdosage-like condition in respect of monensin. Laczay et al. (1990), demonstrating increased activities of microsomal enzymes after simultaneous treatment of chickens with monensin and tiamulin, arrived at a similar conclusion. In our previous experiment, we found significant enhancement of the lipid-peroxidative processes — within a relatively short time — in the liver during acute monensin poisoning of chickens (Sályi et al., 1990).

The aims of the present study were to detect time differences in lipid-peroxidative processes of the liver after salinomycin-tiamulin interaction and salinomycin poisoning, as well as to determine hepatic cytochrome P-450 concentration, in order to obtain a better understanding of the mechanisms of action.

Materials and methods

Acute poisoning was induced in a group of 28-day-old cockerels by oral administration of salinomycin (140 mg/kg body mass) and in another group of birds by oral tiamulin treatment (50 mg/kg body mass). A third group served as control and was treated simultaneously with the same volume of tap-water (0.5 ml per bird). The diet of the birds contained 60 mg/kg salinomycin before and during the experiment. Before treatment and 2, 8 and 24 h thereafter, 6 treated and 6 control chickens each were killed. Their livers were frozen and stored at −18 °C until processed. Before the analyses, the liver samples were homogenized in cold (+4 °C) isotonic saline. Malondialdehyde (TBA-reactive substances) was determined in the intact homogenate using the thiobarbituric acid reaction (Mihara et al., 1980). The glutathione content of the liver homogenate was measured after deproteinization by the method of Sedlak and Lindsay (1968). Cytochrome P-450 (E.C.1.14.14.1) content was measured by the carbon monoxide binding method of Omura and Sato (1964) in the liver microsomal fraction. Glutathione peroxidase (E.C. 1.11.1.9) and catalase (E.C.1.11.1.6) activities of the 10,000 g supernatant of the liver homogenate were determined. Glutathione peroxidase activity was measured using a direct assay (Matkovics et al., 1988) and expressed in a unit that corresponds to 1 nmol glutathione oxidized per minute. Catalase activity was monitored by the assay of Beers and Sizer (1952) and expressed in Bergmeyer units (1 B. U. is the amount of catalase which decomposes 1 meq H_2O_2 per min). The enzyme activities were related to 1 g protein content of the supernatant which was determined using the Folin-phenol reagent (Lowry et al., 1951).

Statistical analysis of the results was done by the paired *t*-test.

Results

Clinical observations

Clinical signs appeared very soon after salinomycin and relatively slowly after tiamulin administration. Two hours after salinomycin treatment, all the treated chickens were sitting or lying on their sides. After tiamulin administration, the onset of clinical signs was slower: about one-third of the birds showed

unsteady gait and signs of depression at 4 h after the treatment. The clinical signs subsided 8 h after the treatment and disappeared 24 h later. No mortality was observed during the experiment.

Biochemical analyses

During the 24-h period of study, a marked increase was found in the malondialdehyde content of the liver, but only in the salinomycin-treated group where the differences were significant at 8 h ($P < 0.05$) and 24 h ($P < 0.01$) after treatment (Fig. 1).

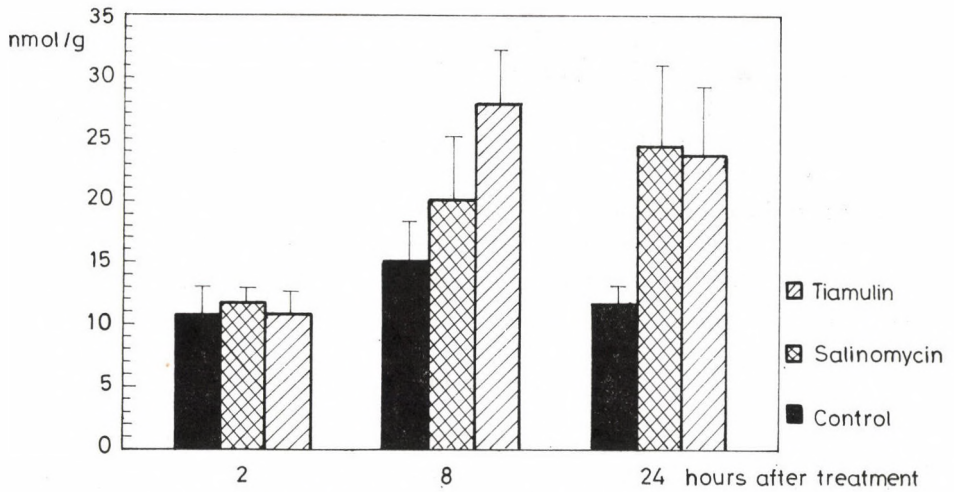

Fig. 1. Glutathione content of the liver homogenate (μmol/g)

Glutathione content of the liver (Fig. 2) showed a significant ($P < 0.001$) decrease in both groups 2 h after treatment and also 8 h after it ($P < 0.001$ in the salinomycin- and $P < 0.05$ in the tiamulin-treated group). One day after the experimental induction of acute salinomycin and tiamulin poisoning, hepatic glutathione concentration rose to the control level.

Cytochrome P-450 concentration of the liver showed a marked increase 8 h after treatment (Fig. 3) but in the tiamulin-treated group the difference was significant ($P < 0.01$) only at that time. One day after intoxication, both treated groups showed a significantly ($P < 0.01$) higher cytochrome P-450 concentration as compared to the control.

Glutathione peroxidase activity was significantly ($P < 0.05$) reduced in both of the treated groups 2 h after treatment, increased to the control level at 8 h, and rose significantly ($P < 0.05$) up to the 24th h in the tiamulin-treated group (Fig. 4). Glutathione peroxidase activity was reduced also in the control group during the period of study.

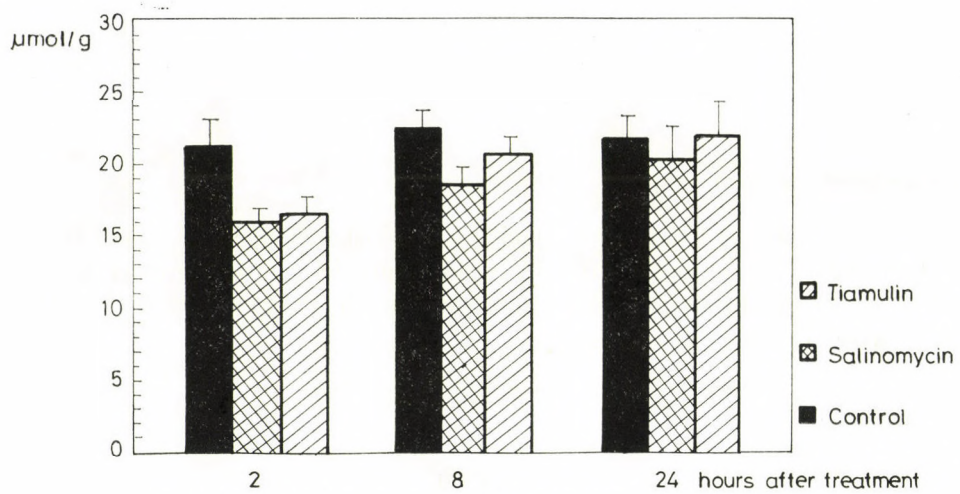

Fig. 2. Malondialdehyde content of the liver homogenate (µmol/g)

Changes in catalase activity were difficult to interpret, since catalase activity was found to decrease rapidly in the control group similarly to glutathione peroxidase, while it remained nearly at the initial level in the treated groups (Fig. 5).

Discussion

The results of this study are rather difficult to interpret; however, they may shed some light on the pathobiochemical events occurring after toxicological interaction between salinomycin and tiamulin, a process that has been observed both in the field and under experimental conditions.

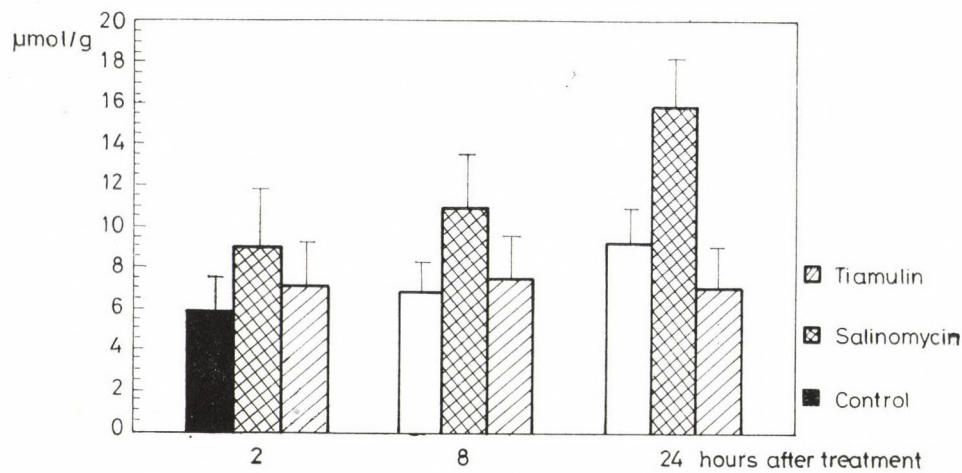

Fig. 3. Cytochrome P-450 content of the liver (nmol/g)

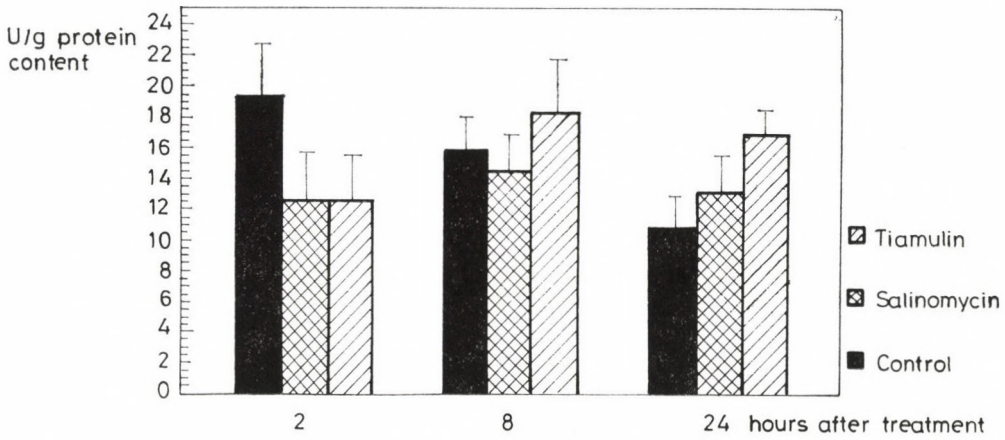

Fig. 4. Glutathione peroxidase activity of the liver homogenate (U/g protein content of 10,000 g supernatant)

The results reported here do not support the previous conclusion (Laczay et al., 1990) that the toxic interaction between tiamulin and ionophore antibiotics is mainly due to an increase in cytochrome P-450 content. Namely, in this study cytochrome P-450 concentration was at the baseline level 2 h after the experimental induction of toxicosis; however, some variables of the biological antioxidative defence mechanism showed pronounced changes. Our results suggest that cytochrome P-450 content is only one of several effects involved in that interaction. The changes of cytochrome P-450 content with

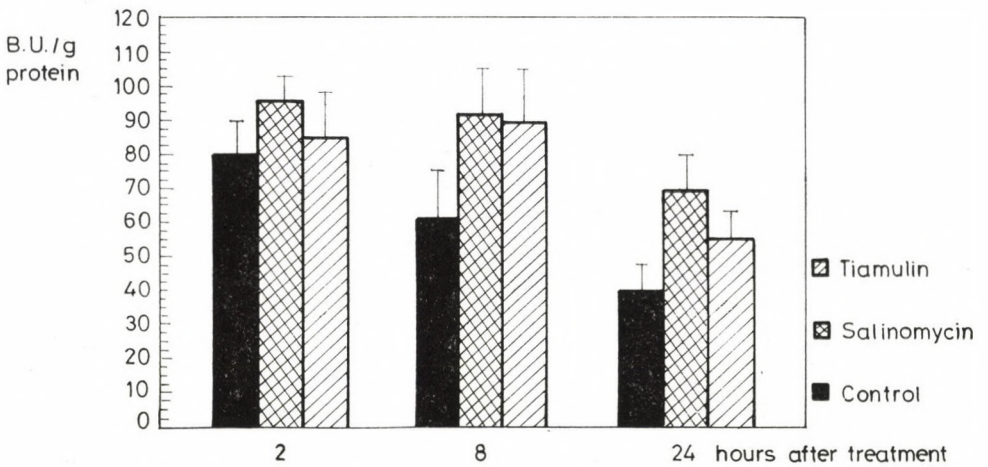

Fig. 5. Catalase activity of the liver homogenate (B. U./g protein content of 10,000 g supernatant)

time are suggestive of an inducing effect of the compounds (salinomycin, tiamulin), which seems to be a phenobarbital-type induction. The observed time-shift is probably due to the fact that membrane-bound and cytosol processes were induced within the mitochondria only after the compounds had passed through the mitochondrial membrane.

A very rapid depletion of reduced glutathione from the liver was found for both drugs. From that observation we may conclude that the incompatibility between ionophore antibiotics and tiamulin is caused by the same mechanism as was found in the case of ionophore poisoning (Sályi et al., 1990).

The experimental protocol of this study was such that the chickens' detoxifying mechanism would be in an activated state at the time of treatment. This was achieved by pretreating the birds with the preventive dose of salinomycin. Another objective was to detect changes in selected biochemical variables with time. This could be reached only by applying a single but large dose of drug, rather than several small doses as is common in the practice. The preventive dose of salinomycin (60 mg/kg of feed) does not cause measurable changes in the parameters of lipid peroxidation and in the activity of the antioxidative defence mechanism. The level of all variables investigated remained in the physiological range found at the beginning of treatment.

Further experiments using different doses of drugs are needed to elucidate some steps of the process which have remained unclear yet.

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THE INVOLVEMENT OF POLYMORPHONUCLEAR LEUKOCYTES IN THE PATHOGENESIS OF BRONCHOPNEUMONIA IN CALVES V. ADHERENCE TO NYLON FIBRES

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The adherence of neutrophils from bronchopneumonic calves to nylon fibres and the influence of this adherence on O_2^- and H_2O_2 production were studied. Polymorphonuclear leukocytes from bronchopneumonic calves were found to produce much more superoxide anion and hydrogen peroxide than neutrophils from healthy animals. A higher production of these compounds was observed in granulocytes adhering to nylon fibres than in cells in suspension. It is suggested that oxygen radicals production induced by the contact of granulocytes with a solid surface plays a role in the destruction of endothelium *in vivo*.

Key words: Calf, bronchopneumonia, granulocyte, adherence

Neutrophils normally circulate within the vasculature as unstimulated cells. However, these cells can become activated in response to stimuli produced within or outside the vascular compartment (Craddock et al., 1977; Ward and Cochrane, 1965). Activated neutrophils release proteolytic enzymes (Janoff, 1975) and produce a series of reactive oxygen species such as superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical (HO*), singlet oxygen ($^1O_2$), and halide- and myeloperoxidase-dependent products of H_2O_2 such as hypochlorous acid (Babior, 1978; Klebanoff, 1980).

The role of oxygen metabolites in neutrophil-dependent endothelial cell injury was first suggested by Sacks et al. (1978). Harlan et al. (1981, 1985) showed that stimulated leukocytes caused endothelial cells to detach from the surface of Petri dishes, and this leukocyte-induced detachment was probably protease mediated. More recent studies by Weiss et al. (1981) and by Martin (1984) have confirmed the ability of reactive oxygen species from activated neutrophils to damage cultured endothelial cells. The hypochlorous acid scavengers provided only limited protection of the endothelium, while azide and cyanide potentiated endothelial damage, suggesting that endothelial cell injury, unlike leukocyte-induced injury of neoplastic cells, was not mediated by myeloperoxidase-generated reactants (Clark and Klebanoff, 1975; Slivka et al., 1980). Hydrogen peroxide is also capable of reacting with transition metals to from the hydroxyl radical. It is suggested that HO* may be

responsible for the antimicrobial action of neutrophils (Murray anc Cohn, 1979; Repine et al., 1981) as well as for the neutrophil-dependent tissue injury associated with immune complex induced vasculitis and systemic activation of complement (Fligiel et al., 1984; Ward et al., 1983).

As inflammation is closely associated with the attachment of leukocytes to solid structures (Neumann and Kownatzki, 1989; O'Flaherty et al., 1977), in this work we report that adherent polymorphonuclear leukocytes differ from suspended cells in their ability to generate superoxide anion and hydrogen peroxide in response to various stimuli.

Materials and methods

The chemotactic formyl peptide (FMLP), calcium ionophore A23187, platelet activating factor (PAF) and cytochrome c were purchased from Sigma (St. Luis, U.S.A.). Leukotriene B_4 (LTB_4) was kindly provided by Dr. J. Rokach, Merck Frosst Canada Inc. (Pointe Claire/Dorval, Canada).

The blood of 40 four-week-old bronchopneumonic calves was used as the source of polymorphonuclear leukocytes, and that of 20 healthy animals of the same age served as control material.

Polymorphonuclear granulocytes were isolated by a combination of dextran sedimentation and density gradient centrifugation on Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) as described by Boyum (1968). The cells were suspended in Hanks' balanced salt solution containing 5 mg/ml of bovine serum albumin. Granulocytes were made adherent on nylon fibres premoistened with the above-mentioned buffer and kept in thermostat at 37 °C. Non-adherent cells were removed immediately by flushing with buffer. The buffer was then replaced with a buffer containing the desired stimulus (FMLP 1×10^{-7} M$\times$l^{-1}; A23187 2.5 μg$\times$ml^{-1}; PAF 5×10^{-6} M$\times$l^{-1}; LTB_4 5×10^{-6} M$\times$l^{-1}) and either cytochrome c 5×10^{-5} M$\times$l^{-1} for measuring superoxide anion production, or 10 mM ferric ammonium sulfate and 2.5 mM potassium thiocyanate for measuring H_2O_2 production (Thurman et al., 1972). To evaluate possible participation of other factors exerted by granulocytes, after stimulation with the above-mentioned agonists the cells were incubated for 30 min with soybean trypsin inhibitor (100 μg$\times$ml^{-1}), O_2^- and H_2O_2 scavengers (superoxide dismutase, 300 IU$\times$ml^{-1} and catalase, 2000 IU$\times$ml^{-1}; one IU of catalase is defined as the amount of enzyme that decomposes 1.0 μM of H_2O_2 per minute at pH 7.0 at 25 °C while the H_2O_2 concentration falls from 10.3 to 9.2 mM), myeloperoxidase inhibitor (sodium azide, 1 μM), HO* scavengers (mannitol, 55 mM and N,N-dimethylthiourea, 10 mM) and ferric ion chelator (deferoxamine mesylate, 100 μM).

Statistical analysis was performed by Student's *t*-test for unpaired data.

Results

Figure 1 shows O_2^- production by nylon fibre adherent neutrophils of healthy calves (white columns) and of bronchopneumonic ones (black columns) in response to various stimuli. Figure 2 shows the same for suspended granulocytes. Fibre-adherent granulocytes produce more superoxide anion than do suspended cells.

Figure 3 shows H_2O_2 production by nylon fibre adherent granulocytes of healthy calves (white columns) and of bronchopneumonic animals (black columns). Figure 4 shows the same for suspended cells. It is clear that the same stimuli which induced an increased O_2^- release also stimulated the release of increased amounts of hydrogen peroxide.

Table 1 summarizes the results obtained with protease inhibitors, O_2^- and H_2O_2 scavengers, myeloperoxidase inhibitor, HO* scavengers and iron chelator. At the concentrations employed, the inhibitors (with the exception of superoxide dismutase, superoxide anion converter to hydrogen peroxide) did not inhibit the generation of superoxide anion. The agents that were protective (dimethylthiourea, mannitol and deferoxamine mesylate) also had an effect on the generation of hydrogen peroxide. Soybean trypsin inhibitor was com-

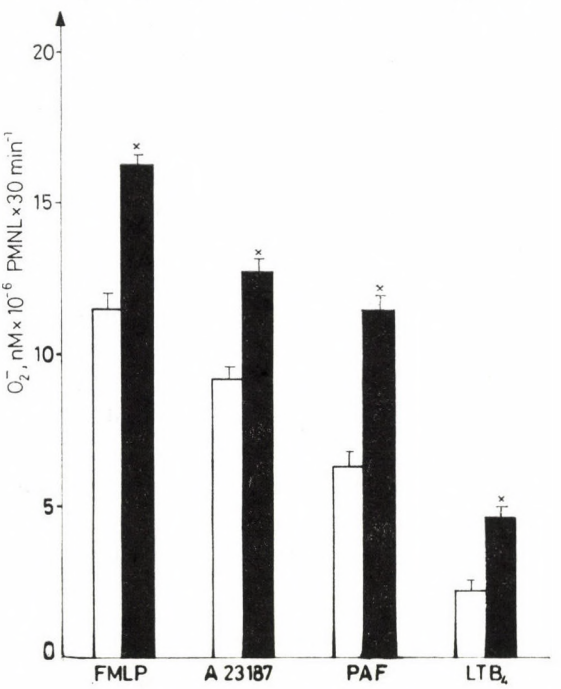

Fig. 1. O_2^- production in nylon fibre adherent neutrophils of healthy (white columns) and bronchopneumonic (black columns) calves. PMNL: polymorphonuclear leukocytes; FMLP: formyl peptide; A23187: calcium ionophore; PAF: platelet activating factor; LTB_4: leukotriene B_4; $^xp < 0.001$

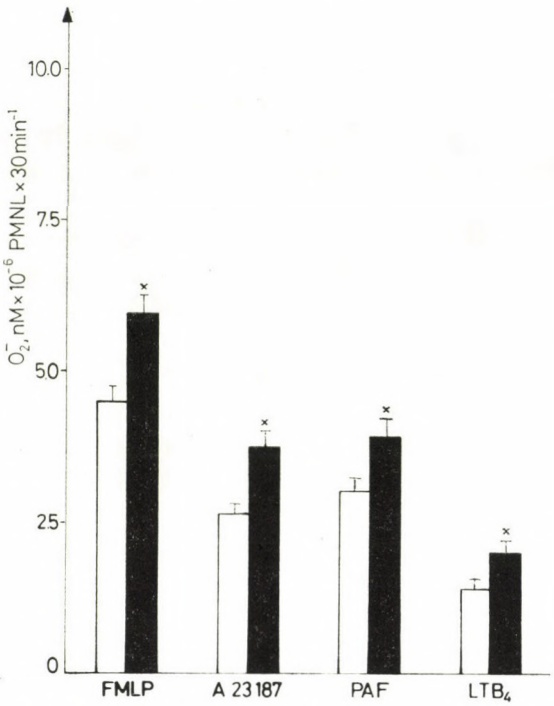

Fig. 2. O_2^- production by neutrophils of healthy (white columns) and bronchopneumonic (black columns) calves in suspension. Other abbreviations as in Fig. 1

pletely ineffective despite the fact that in control studies it significantly inhibited haemoglobin degradation by 12-O-tetradecanoyl phorbol acetate stimulated neutrophils. In the absence of soybean trypsin inhibitor, incubation of 5×10^5 ester-stimulated neutrophils with the haemoglobin substrate caused the production of 53 ± 4 μg of haemoglobin fragments in 18 h (Anson, 1940). The inclusion of 10 or 100 μg soybean trypsin inhibitor in the reaction mixture reduced this amount by 50% and 70%, respectively. Superoxide dismutase was not an effective radical scavenger, whereas catalase provided a significant increase in hydrogen peroxide decomposition. Two hydroxyl radical scavengers (mannitol and dimethylthiourea) and the iron chelator also caused a significant decrease in radical production, but the myeloperoxidase inhibitor did not diminish the production of O_2^- and H_2O_2.

Discussion

Within their lysosomal granules, polymorphonuclear leukocytes contain a number of proteolytic enzymes which may be released in response to inflammatory stimuli (Janoff et al., 1977; Senior et al., 1977). Reactive oxygen metabolites generated from phagocytic cells have been shown to be cytotoxic

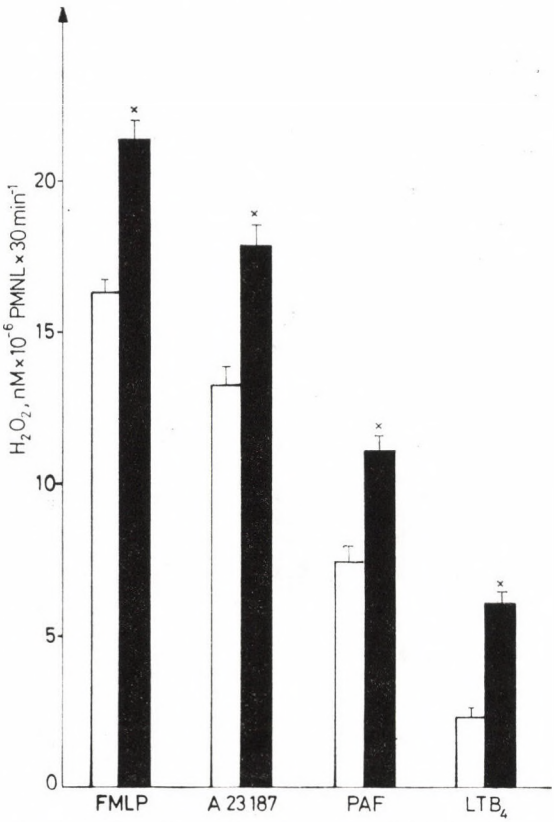

Fig. 3. H_2O_2 production in nylon fibre adherent neutrophils of healthy (white columns) and bronchopneumonic (black columns) calves. Other abbreviations as in Fig. 1

to a variety of cells including erythrocytes, leukocytes, platelets, neoplastic cells, fibroblasts and endothelial cells (Baehner et al., 1977; Nathan et al., 1979; Simon et al., 1981). In recent years, substantial evidence has been accumulated to suggest that oxygen metabolites derived from inflammatory cells may be responsible for the acute injury of tissue (Fantone and Ward, 1982).

We have shown that neutrophils of bronchopneumonic calves produce a larger amount of reactive oxygen species as compared to those of healthy calves, and that adherent cells produce more oxygen radicals than do granulocytes in suspension. Similar results have been obtained by Kownatzki and Uhrich (1987). These authors have shown that in response to certain stimuli nylon fibre adherent polymorphonuclear leukocytes produce considerably higher amounts of superoxide anion than do granulocytes in suspension. We have shown that the production of these compounds may be significantly reduced by catalase but not by protease inhibitor. This findings is consistent with the results obtained by Martin (1984) and Sacks et al. (1978). In agree-

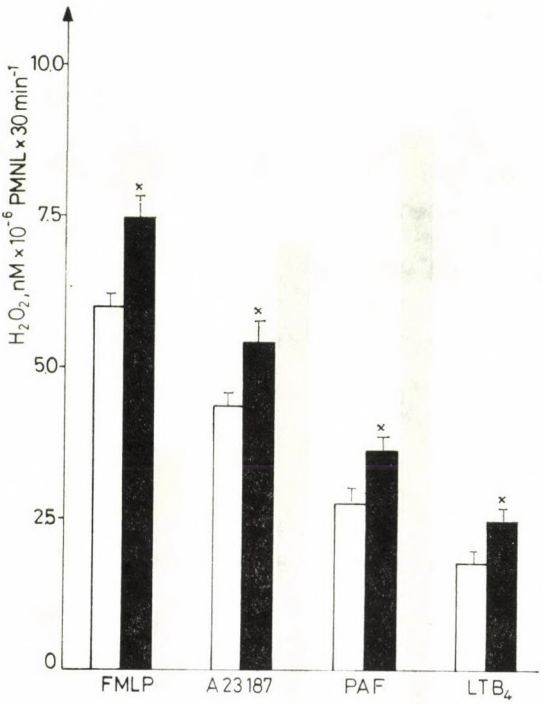

Fig. 4. H_2O_2 production by neutrophils of healthy (white columns) and bronchopneumonic (black columns) calves in suspension. Other abbreviations as in Fig. 1

Table 1

The influence of inhibitors on O_2^- and H_2O_2 production (nM$\times 10^6$ cells/hour) in nylon fibre adherent polymorphonuclear leukocytes of calves

	Healthy		Diseased	
	O_2^-	H_2O_2	O_2^-	H_2O_2
No inhibitor	51±3	22±1	84±4	37±2
Soybean trypsin inhibitor	50±3	—	82±3	—
Superoxide dismutase	—	—	—	—
Catalase	57±2	—	88±4	—
Dimethylthiourea	61±4	21±2	91±6	38±3
Mannitol	60±4	24±2	90±5	41±3
Deferoxamine mesylate	50±4	21±2	82±3	36±3
Sodium azide	51±3	—	83±4	—

ment with the results of Fox (1984) and Halliwell and Gutteridge (1984), we have also shown that dimethylthiourea, mannitol and deferoxamine mesylate decrease O_2^- and H_2O_2 production.

As all cells that have entered the tissues may be considered adherent to solid structures, the results of this work suggest that this phenomenon may be partly responsible for oxygen metabolite generation by granulocytes in inflammatory conditions.

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THE INVOLVEMENT OF POLYMORPHONUCLEAR LEUKOCYTES IN THE PATHOGENESIS OF BRONCHOPNEUMONIA IN CALVES VI. SUPEROXIDE DISMUTASE AND LIPOPROTEIN LIPASE ACTIVITIES

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The kinetics of superoxide anion production in polymorphonuclear leukocytes of bronchopneumonic calves, as well as superoxide dismutase and lipoprotein lipase activities in the blood plasma of these animals were studied. Granulocytes of bronchopneumonic calves were found to produce 10 times as much O_2^- radicals as those of healthy animals. Superoxide dismutase and lipoprotein lipase activities were lower in the plasma of bronchopneumonic calves. *Escherichia coli* endotoxin was found to stimulate superoxide anion production in polymorphonuclear leukocytes in a dose-dependent manner. These facts suggest that an endogenous mediator, probably cachectin or some other monokine(s), plays a role in granulocyte activation during calf bronchopneumonia.

Key words: Calf, bronchopneumonia, superoxide dismutase, lipoprotein lipase, endotoxin, neutrophils

Polymorphonuclear leukocytes have been implicated in the aetiology of adult respiratory distress syndrome (Heflin and Brigham, 1981) through the production and extracellular release of toxic oxygen metabolites (Zimmermann et al., 1983). The production of these metabolites is physiologically necessary as the major mechanism used by neutrophils to kill bacteria (Klebanoff, 1982). However, their overproduction in inflammatory conditions can be the cause of bystander cell and tissue injury (Johnson et al., 1981; Martin et al., 1981; Sacks et al., 1978; Shasby et al., 1983). Superoxide anion, a major neutrophil oxidant, is formed during the respiratory burst of neutrophils in response to a variety of physiologic and nonphysiologic conditions. These conditions include the phagocytosis of bacteria and yeasts such as opsonized zymosan (Curnutte et al., 1975; Drath and Karnovsky, 1975; Root and Metcalf, 1977), phorbol myristate acetate, a semisynthetic, nonspecific maximal cell stimulator *via* protein kinase C activation (De Chatelet et al., 1980; Lehrer and Cohen, 1981; Newburger et al., 1980), and N-formyl-methionyl-leucyl-phenylalanine (FMLP), a synthetic analogue of uniquely formylated bacterial peptides (Becker et al., 1979; English et al., 1981; Simchowitz et al., 1979).

Superoxide anion may be directly toxic to cells, either as a reducing or oxidizing agent or *via* conversion to other toxic oxygen metabolites such as hydrogen peroxide and hydroxyl radical (Southorn and Powis, 1989).

The aim of this study was to examine the kinetics of superoxide anion production in polymorphonuclear leukocytes of bronchopneumonic calves. The influence of *Escherichia coli* endotoxin on the production of superoxide anion in granulocytes was also examined, and the activity of superoxide dismutase activity in erythrocytes and that of lipoprotein lipase in the blood plasma were studied.

Materials and methods

The blood of 40 bronchopneumonic calves (age: 4 weeks) was used as the source of polymorphonuclear leukocytes and erythrocytes. The blood of 20 healthy calves of the same age was used as control material.

Neutrophils were isolated according to Boyum (1968) and were stimulated with opsonized zymosan. Superoxide anion production was assayed by the method of cytochrome c reduction (Pick, 1986).

Endotoxin concentration of the blood plasma was estimated using Coatest Endotoxin Kit (Kabi Vitrum, Mölndäl, Sweden).

Gram-negative bacterial endotoxins catalyse the activation of an enzyme system in the amoebocytes of the horseshoe crab, *Limulus polyphemus*. The activated enzyme catalyzes the splitting of p-nitroaniline (pNA) from the synthetic substrate Ac-Ile-Glu-Gly-Arg-pNA. After stopping the reaction with acetic acid, the rate at which pNA has been released, is measured photometrically at 405 nm.

$$\text{Proenzyme} \xrightarrow{\text{Endotoxin}} \text{Enzyme}$$

$$\text{Ac-Ile-Glu-Gly-Arg-pNA} \xrightarrow{\text{Enzyme}} \text{Ac-Ile-Glu-Gly-Arg} + \text{pNA}$$

O_2^- production in granulocytes under the influence of endotoxin

A granulocyte suspension (1×10^6, cells $\times$ ml^{-1}) was incubated with opsonized zymosan (1 mg $\times$ ml^{-1}). To the incubation mixture, *E. coli* endotoxin (type 0111:B_4, Difco, Corp., Detroit, U.S.A.) was added in concentrations of 1 μg/ml, 5 μg/ml and 10 μg/ml. After 20, 40 and 60 min incubation, the cells were centrifuged and O_2^- concentration was estimated in the supernatant according to Johnson et al. (1975). The results were expressed as nM reduced cytochrome c per min, assuming an extinction coefficient of 1.96×10^4 M^{-1} cm^{-1} (Yonetani, 1965).

Superoxide dismutase activity in red blood cells

Fifty μl of blood was haemolysed by mixing with 1950 μl of cold (4 °C) water, and the haemoglobin was extracted into 0.5 ml of chloroform-ethanol (3 : 5 v/v) mixture by vigorous mixing (Minami and Yoshikawa, 1979). The mixture was centrifuged (30 min, 1500 g, 4 °C) and the superoxide dismutase activity and superoxide anion concentration of the supernatant were determined.

The reaction mixture contained xanthine (100 μM/ml), 150 μg of bovine serum albumin, nitroblue tetrazolium (25 μM/l), and 0.1 ml of supernatant, brought to a final volume of 3 ml with sodium carbonate buffer (50 mM, pH 10.2) containing 100 μM EDTA. After preincubating the reaction mixture for 10 min at 25 °C, 30 μg of xanthine oxidase was added and the mixture was incubated for another 20 min. The reaction was stopped by adding 0.1 ml of 6 mM $CaCl_2$. The increase in extinction at 560 nm was measured after 20 min. One unit of superoxide dismutase activity is defined as the amount of enzyme required to inhibit nitroblue tetrazolium reduction by 50% (McCord and Fridovich, 1969).

Other estimations

Superoxide anion concentration was measured by preincubating 0.1 ml of supernatant with 1 ml of 50 mM phosphate buffer (pH 7.4) containing glucose (2 g/l) and bovine serum albumin (2 g/l), with or without superoxide dismutase, for 5 min at 37 °C. To initiate the reaction, 0.1 ml of 1.2 mM cytochrome c solution was added, and the increase in absorbance at 550 nm was measured (Johnston et al., 1975). Lipoprotein lipase activity was measured as described previously (Ledwożyw, 1986).

Statistical analyses were performed by Student's *t*-test for unpaired data.

Results

Table 1 shows O_2^- production in polymorphonuclear leukocytes in response to zymosan stimulation. Neutrophils of bronchopneumonic calves produce 10 times more of this radical than do granulocytes of control animals.

Figure 1 shows the time-dependence of O_2^- production in unstimulated granulocytes of calves. A high production of superoxide anion in granulocytes of diseased animals is evident.

Figure 2 shows the time-dependence of O_2^- production in zymosan-stimulated granulocytes. Neutrophils of bronchopneumonic calves produce

Table 1

Superoxide anion production in granulocytes (nM/1×10^6 cells) in response to 30-min zymosan stimulation

	Unstimulated	Stimulated
Healthy (A)	1.80±0.21+	27.22±1.25
Bronchopneumonic (B)	18.70±1.24*	39.64±1.86*

+mean values ± SD; Student's *t*-test for unpaired data: *p < 0.001 B vs A

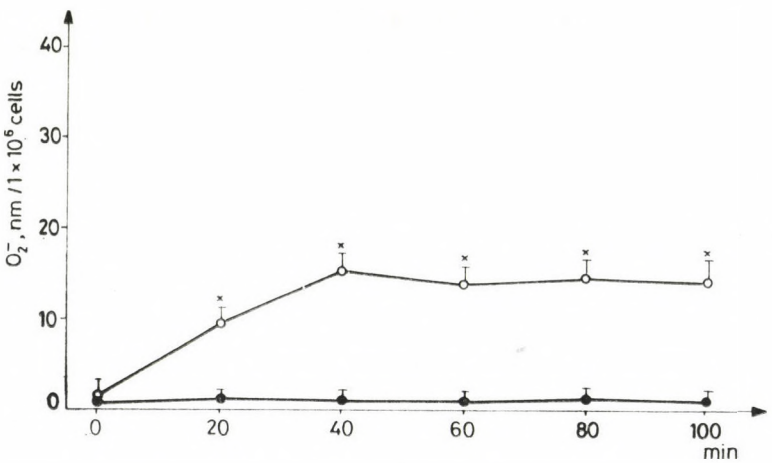

Fig. 1. Time-dependence of O_2^- production in unstimulated granulocytes of calves. ● — granulocytes of healthy calves; ○ — granulocytes of bronchopneumonic calves; *p < 0.001

Table 2

Superoxide dismutase activity (units per mg protein) and O_2^- concentration (nM of reduced cytochrome c×min^{-1}×mg $protein^{-1}$) in calf blood

	Superoxide dismutase	O_2^-
Healthy (A)	9.35±0.86+	0.120±0.010
Bronchopneumonic (B)	4.51±0.42*	0.230±0.020*

+mean values ± SD; Student's *t*-test for unpaired data: *p < 0.001 B vs A

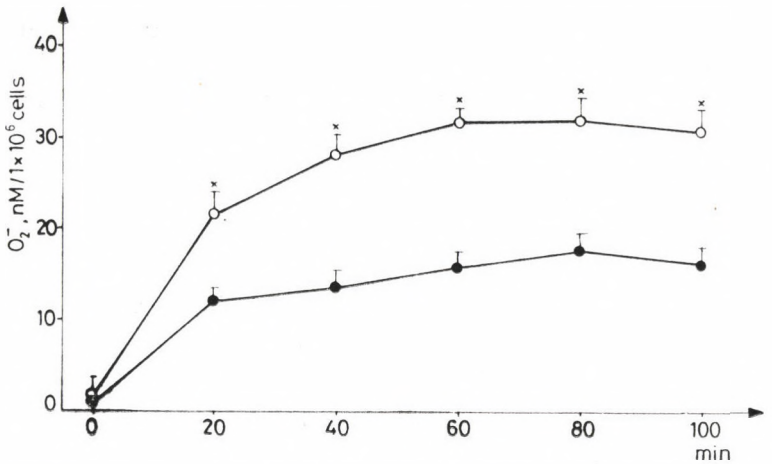

Fig. 2. Time-dependence of O_2^- production in zymosan-stimulated granulocytes of calves. ● — granulocytes of healthy calves; ○ — granulocytes of bronchopneumonic calves; *$p < 0.001$

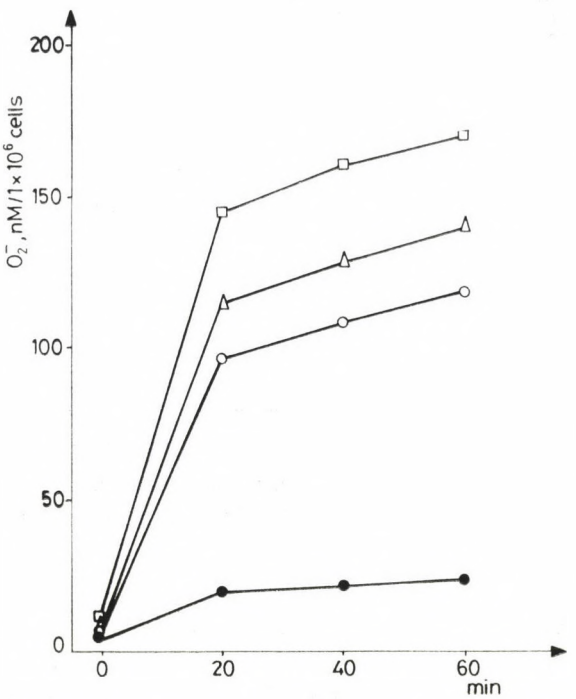

Fig. 3. The influence of *Escherichia coli* endotoxin on O_2^- production in unstimulated granulocytes of healthy calves. *E. coli endotoxin:* ○ — 1 μg/ml; △ — 5 μg/ml; □ — 10 μg/ml; ● — without endotoxin

larger amounts of O_2^- than do granulocytes of healthy animals and unstimulated granulocytes of both groups.

Table 2 shows superoxide dismutase activity and O_2^- concentration in the blood of calves. Bronchopneumonic animals showed a fall in superoxide dismutase activity.

Figure 3 illustrates the influence of *E. coli* endotoxin on superoxide anion production in granulocytes of healthy calves. The production of this radical depends on the endotoxin concentration and the time of incubation.

Figure 4 shows the influence of *E. coli* endotoxin on O_2^- production in nonstimulated neutrophils of bronchopneumonic calves. The incubation of neutrophils with endotoxin caused a rise in O_2^- production.

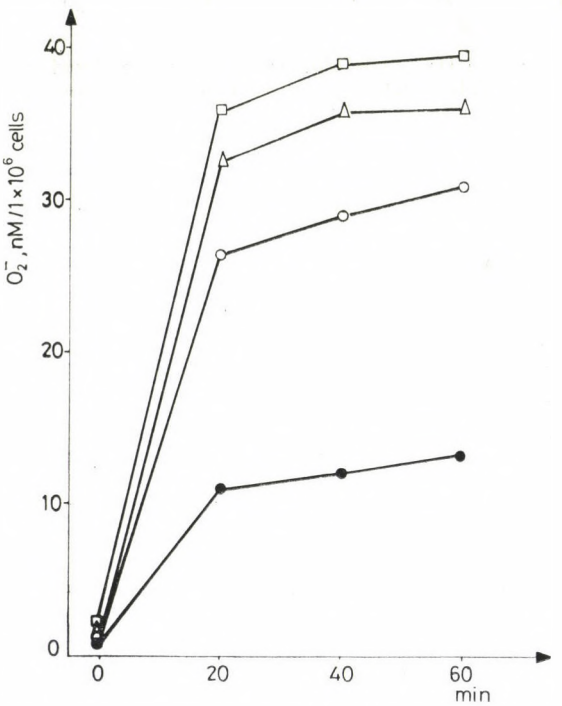

Fig. 4. The influence of *Escherichia coli* endotoxin on O_2^- production in unstimulated granulocytes of diseased calves. *E. coli* endotoxin: ○ — 1 μg/ml; △ — 5 μg/ml; □ — 10 μg/ml; ● — without endotoxin

Figure 5 shows the influence of *E. coli* endotoxin on O_2^- production in zymosan-stimulated neutrophils of healthy calves. Figure 6 illustrates the same for bronchopneumonic animals. The profile of O_2^- production is similar to that observed in unstimulated granulocytes; however, the production of superoxide anion is higher in both groups.

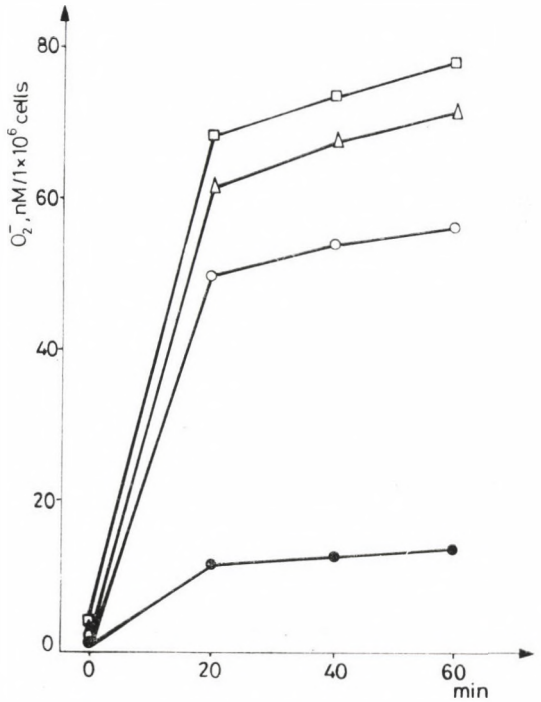

Fig. 5. The influence of *Escherichia coli* endotoxin on O_2^- production in zymosan-stimulated granulocytes of healthy calves. *E. coli* endotoxin: ○ — 1 μg/ml; △ — 5 μg/ml; □ — 10 μg/ml; ● — without endotoxin

Table 3 summarizes the endotoxin concentrations and lipoprotein lipase activities in the blood plasma. Endotoxin concentration was by about 290% higher in bronchopneumonic calves than in the control group. However, lipoprotein lipase activity was lower in the plasma of bronchopneumonic calves.

Table 3

Endotoxin concentration (ng/ml) and lipoprotein lipase activity (nM × ml^{-1}) in the blood plasma of calves

	Healthy (A)	Diseased (B)
Endotoxin	5.1±0.4+	19.8±1.1*
Lipoprotein lipase	4.4±0.3	2.0±0.2*

+mean values ± SD; Student's *t*-test for unpaired data: *$p < 0.001$

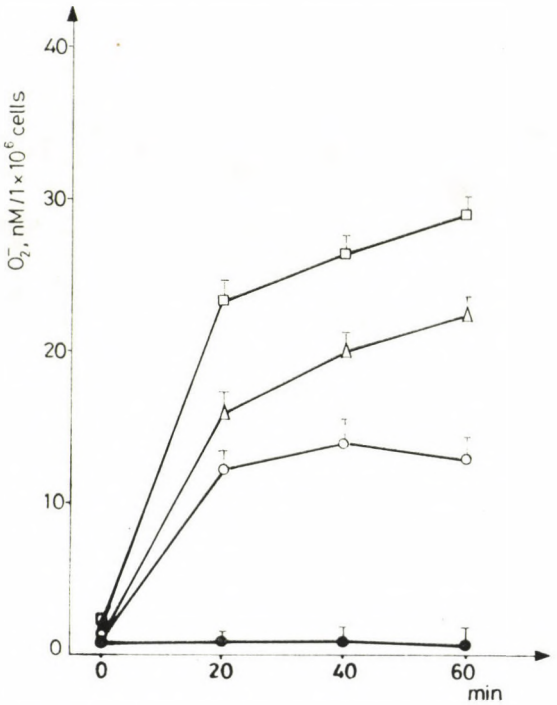

Fig. 6. The influence of *Escherichia coli* endotoxin on O_2^- production in zymosan-stimulated granulocytes of diseased calves. *E. coli* endotoxin: ○ — 1 μg/ml; △ — 5 μg/ml; □ — 10 μg/ml; ● — without endotoxin

Discussion

Various disease states are connected with the defects of physiological functions of granulocytes. Circulating neutrophils of endotoxaemic rats are characterized by a markedly increased production of superoxide (Holman and Maier, 1988), with a concomitant decreased response to a phagocytic stimulus such as zymosan. This defect may account for the increased risk of pulmonary sepsis in adult respiratory distress syndrome (Montgomery et al., 1985).

An analogous situation can be observed in patients with chronic granulomatous disease, in which neutrophils cannot generate oxygen metabolites in a quantity sufficient to kill catalase-containing bacteria (Baehner et al., 1972; Tauber et al., 1983).

Similar changes have been observed by Tennenberg and Solomkin (1988) in superoxide anion production in granulocytes of septic patients. Other authors have observed depression of myeloperoxidase activity in endotoxaemic granulocytes (Simons et al., 1987). Adherence to both coverslips and a standard nylon wool column, as well as phagocytosis of yeast particles in this model has been shown to be depressed (Griswold and Maier, 1988). Chemotaxis of neutro-

phils to phagocytize latex beads was also depressed in this model of adult respiratory distress syndrome (Heflin and Brigham, 1981). Similar findings have been reported from humans suffering from intra-abdominal infection Alexander et al., 1979; Solomkin et al., 1981).

Defects in host defence mechanisms enhance the risk of infection (Bode et al., 1974; Neu, 1984; Peterson, 1984). Considerable progress has been made in defining both the nonspecific and specific reactions of the host's defence to infection (Babior, 1978; Badwey and Karnovsky, 1980; Marx, 1983), but little is known about the relationship between these mechanisms and oxygen free radicals or their scavengers.

Animals infected with various bacteria, parasites or viruses develop a catabolic state that can advance to severe cachexia (wasting), shock and death. These animals exhibit marked lipaemia in the last few weeks of the disease. Investigations into the mechanism of this paradoxal hypertriglyceridaemia revealed a deficiency in lipoprotein lipase, an enzyme essential for clearing triglyceride from the blood (Rouzer and Cerami, 1980). The first indication that an endogenous mediator caused the loss of lipoprotein lipase activity has been observed by Kawakami and Cerami (1981). This factor could be produced *in vitro* by endotoxin treatment of macrophages (Beutler et al., 1985). Other key lipogenic enzymes including acetyl-CoA carboxylase, fatty acid synthetase (Pekala et al., 1983) and glycerophosphate dehydrogenase (Torti et al., 1985) were suppressed by this endotoxin-induced monokine. This factor, later named cachectin, is one of the major secretory proteins produced by the macrophage in response to endotoxin. The ability of neutrophils to phagocytize latex beads and lyse antibody-coated chicken erythrocytes is significantly augmented by cachectin. Cachectin also promotes the adherence of neutrophils to endothelial cells (Gamble et al., 1985; Shalaby et al., 1985) and the migration and diapedesis of neutrophils (Dinarello et al., 1986; Silberstein and David, 1986).

The fall observed by us in lipoprotein lipase activity of the blood plasma of bronchopneumonic animals may suggest the existence of similar mechanisms involving cachectin action in the pathogenesis of calf bronchopneumonia.

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THE INVOLVEMENT OF POLYMORPHONUCLEAR LEUKOCYTES IN THE PATHOGENESIS OF BRONCHOPNEUMONIA IN CALVES VII. GRANULOCYTE ACTIVATION BY ARACHIDONIC ACID

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Arachidonic acid-induced granulocyte activation was studied in calves. Both in healthy and in bronchopneumonic animals, neutrophil stimulation with 67 μM arachidonic acid caused a rise in granulocyte exocytosis. This event, however, was much more intense in granulocytes from diseased calves. Fatty acids were found to stimulate granule exocytosis from granulocytes. Oleic acid possessed the weakest while dihomo-γ-linoleic acid showed the strongest activity of this type. It is suggested that arachidonic acid may be an important mediator of calf bronchopneumonia.

Key words: Calf, bronchopneumonia, neutrophil activation, arachidonic acid

One of the signs indicative of inflammation is the presence of neutrophils releasing their granule constituents into the surrounding tissues in response to various stimuli. The mechanism of stimulus-response coupling described for granulocyte activation involves the hydrolysis of phosphatidylonositol 4,5-biphosphate (Berridge, 1984; Smith et al., 1985; Smith et al., 1986), which results in the generation of two second messengers, inositol-1,4,5-triphosphate and 1,2-diacylglycerol (Berridge, 1984; Berridge and Irvine, 1984). These messengers mediate the mobilization of intracellular Ca^{2+} (Dougherty et al., 1984; Prentki et al., 1984) and phospholipid-dependent protein kinase C activation (Nishizuka, 1984). Neutrophils metabolize arachidonic acid, by the lipoxygenase pathway, to hydroxyeicosatetraenoic acid (HETEs) and leukotriene B_4 (Payan et al., 1984) which stimulates these cells to express various functions (Smith et al., 1984).

Lipoxygenase inhibitors have been shown to suppress neutrophil activation (Duque et al., 1986; Naccache et al., 1979; Smith et al., 1982; Smith et al., 1986; Smolen and Weissman, 1980), and products of arachidonic acid lipoxygenation have also been implicated in neutrophil activation. However, arachidonic acid itself has been demonstrated to induce neutrophil aggregation (O'Flaherty et al., 1979) and oxygen radical production (Badwey et al., 1981; Badwey et al., 1984).

In view of the above findings, in this work we have studied the influence of arachidonic acid on neutrophil degranulation in bronchopneumonic calves.

Materials and methods

Blood from 40 four-week-old bronchopneumonic calves was used as the source of polymorphonuclear leukocytes, and that from 20 healthy animals of the same age served as control.

Polymorphonuclear leukocytes were obtained by dextran sedimentation and centrifugation on Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) according to Boyum (1968). The cells were suspended in a buffer containing 138 mM NaCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 2.7 mM KCl, 0.6 mM $CaCl_2$, 1.0 mM $MgCl_2$ and 5.55 mM glucose, and incubated according to various procedures described in the Results. After incubation, the samples were centrifuged at 1,000 g for 10 min and the supernatant was assayed for myeloperoxidase and lactate dehydrogenase activities according to Henson et al. (1978) and Bergmeyer et al. (1965), respectively.

Statistical analyses were performed by Student's *t*-test for unpaired data.

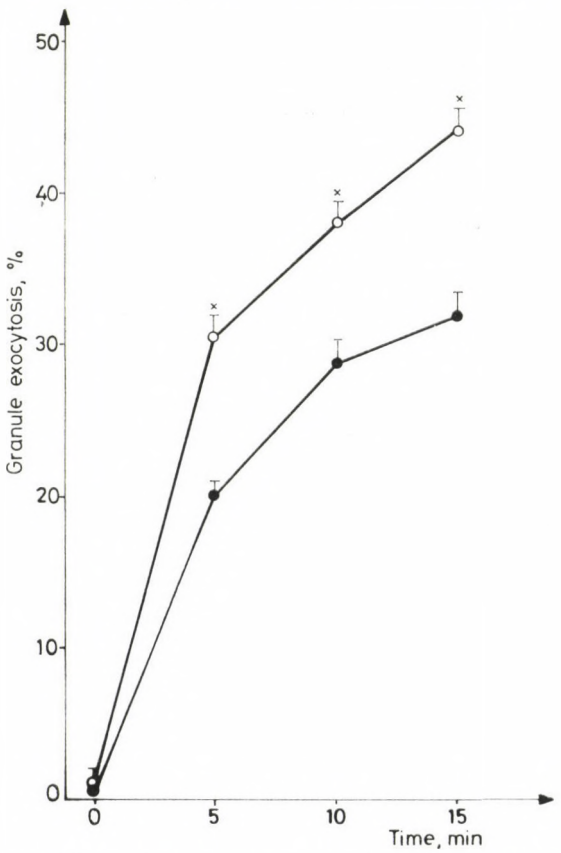

Fig. 1. Arachidonic acid-induced myeloperoxidase release from granulocytes as a function of stimulation time. ● — granulocytes from healthy calves; ○ — granulocytes from diseased calves; x = $p < 0.001$

Results

Figure 1 shows that arachidonic acid stimulates myeloperoxidase release from neutrophils. Stimulation with 67 μM arachidonic acid caused a time-dependent increase of granulocyte exocytosis. In granulocytes from bronchopneumonic calves, degranulation was more expressed that in those from healthy animals.

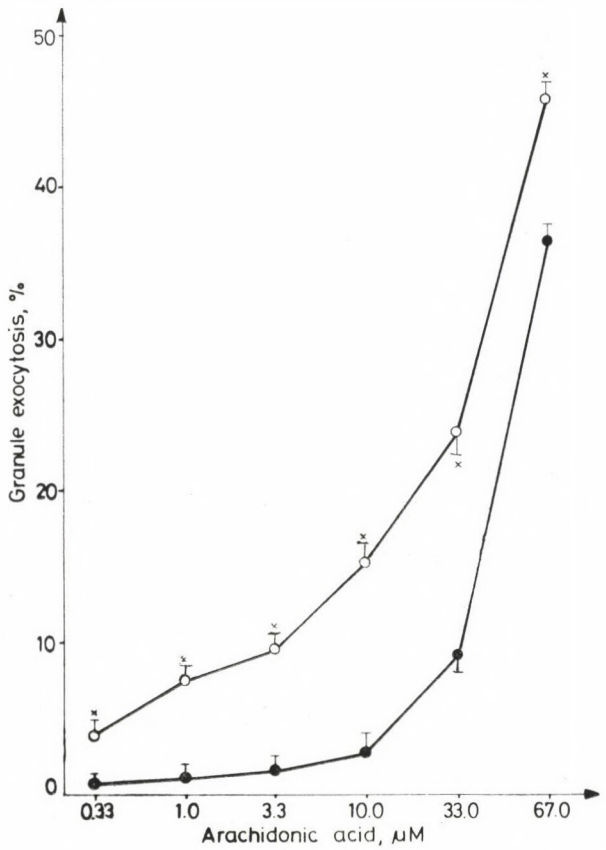

Fig. 2. Arachidonic acid-induced myeloperoxidase release from granulocytes as a function of arachidonic acid concentration. ● — granulocytes from healthy calves; ○ — granulocytes from diseased calves; x = $p < 0.001$

Figure 2 shows a concentration-dependent stimulation of myeloperoxidase release by arachidonic acid. The degranulation process was much more intense in granulocytes from bronchopneumonic calves.

Degranulation has been shown to be a specific process in both cases (no lactate dehydrogenase release).

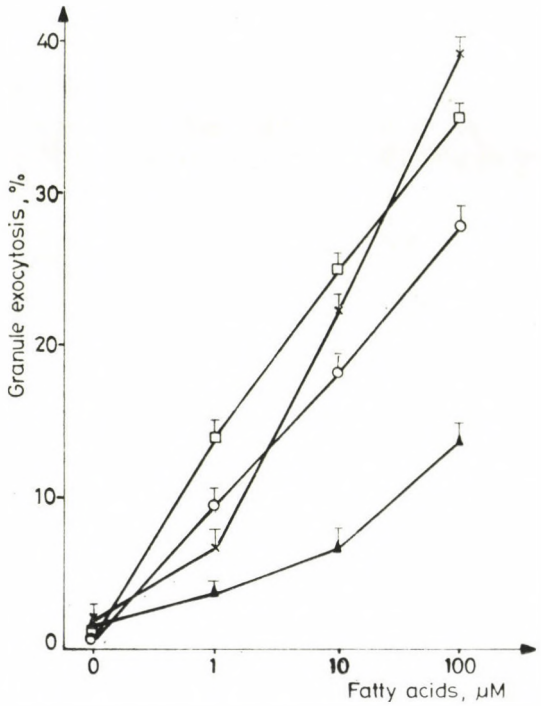

Fig. 3. Influence of fatty acids on granule exocytosis from the neutrophils of healthy calves. □ — dihomo-τ-linoleic acid; ▲ — oleic acid; x — linolenic acid; ○ — linoleic acid

Figure 3 shows the influence of cis-unsaturated fatty acid on granule exocytosis from granulocytes. The weakest effect was observed in the case of oleic acid while the strongest one for dihomo-γ-linoleic acid.

Discussion

In this work, we have shown that arachidonic acid elicits granule exocytosis from the neutrophils of calves. In contrast to phorbol myristate acetate which induces the exocytosis of only specific granules (Smith and Iden, 1979), arachidonic acid stimulates the release of both azurophilic and specific granule constituents.

Cis-polyunsaturated fatty acids were found to stimulate neutrophil degranulation, and the respective secretory capacities were correlated with the degree of unsaturation. The requirement for unsaturation relative to secretory capacity is emphasized by the finding of Smith et al. (1987), i.e. that saturated fatty acids do not stimulate granule exocytosis from neutrophils. The ability of fatty acids to stimulate PMNs as a function of the unsaturation degree has also been reported for superoxide anion production (Badwey et al., 1981).

Polymorphonuclear leukocytes exposed to arachidonic acid generate 5- and 15-HETE (Payan et al., 1984), and 5-HETE has been reported to stimulate polymorphonuclear granulocyte degranulation (Stenson and Parker, 1980). It is conceivable, therefore, that arachidonic acid-induced PMNL activation is mediated by metabolites of the lipoxygenase pathway. Lipoxygenase inhibitors have been found, however, to esert no effect on granule exocytosis from arachidonic acid-stimulated PMNL (Smith et al., 1987).

The data presented in this work, together with the observation that arachidonic acid can induce an inflammatory reaction associated with PMNL infiltration (Carlson et al., 1985; Chang et al., 1986), suggest that arachidonate is an important inflammatory mediator in calf bronchopneumonia, stimulating granulocyte exocytosis.

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ACTIVITY OF SERUM CREATINE KINASE, LACTATE DEHYDROGENASE AND LD ISOENZYMES IN PIGLETS AFFECTED WITH CONGENITAL TREMOR: A CASE REPORT

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The activities of serum creatine kinase (CK), lactate dehydrogenase (LD) and LD isoenzymes were studied in 14 Prestice black pied pigs from a herd affected with congenital tremor. Mean CK activity was 19.57 $\pm$ 3.56 μkat $\cdot$ l^{-1} for 6 adult pigs, and it was 21.03 $\pm$ 1.33 μkat $\cdot$ l^{-1} and 20.42 $\pm$ 1.23 μkat $\cdot$ l^{-1} for the affected (n = 5) and control (n = 3) piglets, respectively. No significant differences were demonstrable between the groups in CK activity. Total serum LD and LD-4 as well as LD-5 isoenzyme activities were higher in sows. Piglets affected with congenital tremor showed an increase in total LD enzyme and LD-5 isoenzyme activity. It is concluded that no relationship exists between congenital tremor and serum CK activity in piglets. At the same time, there is a positive relationship between congenital tremor and significantly ($P < 0.01$) elevated LD enzyme and LD-5 isoenzyme activity. The results allow us to suggest that total lactate dehydrogenase and LD-5 isoenzyme activities could be used as biological markers of congenital tremor in piglets.

Key words: Congenital tremor, piglet, creatine kinase, lactate dehydrogenase, isoenzymes

Intensive selection for meatiness in pigs has resulted in an increased incidence of various neuromuscular diseases including congenital tremor in piglets.

Little is known about the primary biochemical changes that initiate a cascade resulting in muscle cramps, fasciculations, and altered cell membrane permeability, the signs of this disorder. A selective increase in membrane permeability, caused by alterations in the structure and function of cellular membranes, helps to explain the leakage of certain soluble cytoplasmic enzymes into the extracellular fluid (Doize et al., 1990; Duthie et al., 1989; Gupta et al., 1991; Roodenburg et al., 1990). Enzymologic data on these enzymes, especially creatine kinase and lactate dehydrogenase, appearing in increased levels in the blood of animals with myopathies, are occasionally found in the literature (DiBartola and Tasker, 1977; Hoven et al., 1988; Rozkosna et al., 1986; Roodenburg et al., 1990), but no data have been reported yet from piglets with congenital tremor.

Creatine kinase (CK, EC 2.7.3.2) is an organ-specific cytoplasmic enzyme predominantly present in the skeletal muscle, heart and brain. The enzyme is located in the mitochondria where ATP is produced or consumed, and catalyzes

the transfer of energy-rich phosphates to the phosphate-carrier creatine in order to form creatine phosphate. Located at the ATP-using side, CK is involved in the formation of ATP by a transfer of the energy-rich phosphate group from creatine phosphate to ADP. In total, there is a creatine phosphate shuttle which serves as an initial short-term energy source for the muscle (Roodenburg et al., 1990).

Lactate dehydrogenase (LD, EC 1.1.1.27), another cytoplasmic enzyme, plays an important role in the glycolytic cycle, and is therefore crucially important for normal muscle physiology (Gupta et al., 1991). LD is present in virtually all tissues. Consequently, elevations of the total enzyme activity in serum reflect no specific damage of a single tissue or organ (Maekawa, 1988). Additional information can be obtained through the separation and quantitation of LD isoenzymes composed of four subunits of either the M (muscle) or the H (heart) type. The five isoenzymes are numbered MMMM (LD-5), MMMH (LD-4), MMHH (LD-3), MHHH (LD-2) and HHHH (LD-1). LD-5 and LD-4 are associated with the anaerobic while LD-1 and LD-2 with the aerobic pathways of carbohydrate metabolism (Doize et al., 1989), whereas LD-3 is not strictly classified in either of these categories.

This study was designed to determine the benefit and potential costs of LD and CK enzymes and LD isoenzymes in the diagnosis of congenital tremor in piglets, and to correlate them with the clinical ,aetiological, pathological and epidemiological findings (Tomko et al., 1991).

Materials and methods

The activity of CK, LD and LD isoenzymes was determined in the blood serum obtained from 14 Prestice black pied pigs (8 piglets, 3 sows, 3 boars) of a herd affected with congenital tremor.

The piglets were between 5 and 20 days old, while the sows and boars were 1- to 3-year-old breeding animals maintained on a breeding farm in East Slovakia. All adult animals were fed standard industrial feeds for lactating sows and breeding boars, and had uniform access to water.

Samples were collected from five piglets of two litters with evidence of congenital tremor and from a normal healthy litter with a total of three piglets. The parents of the tested piglets (boars and sows) had no history and showed no clinical signs of the disease. Blood samples were obtained on five separate occasions from the anterior vena cava in snout-restrained sows and boars, and from the ophthalmic sinus in piglets. All samples were centrifuged upon receipt and the serum was separated into three aliquots until analysis, which always took place within 24 h. The aliquots were used for measuring total CK and LD activity and for determining LD isoenzyme patterns.

Total CK activity in serum was measured by a CK NAC-activated method (Boehringer, Mannheim). Activity was measured at 25 °C in an enzymatic system where ATP produced from creatine phosphate was utilized for the production of glucose-6-phosphate, which was then oxidized to 6-phosphogluconate under simultaneous reduction of NADPH. The reduction of NADPH was monitored spectrophotometrically at 365 nm.

Total LD activity in serum was determined spectrophotometrically (510 nm) by the reduction of NAD + to NADH at 25 °C, using a commercial kit (Bio-Lachema, Brno, Czecho-Slovakia).

LD izoenzymes were separated by a disc-polyacrylamide gel electrophoresis by the method of Dietz and Lubrano (1967) (cit. by Knudsen et al., 1970). Electrophoresis was performed at 50 volts for 60 min with a current not exceeding 2.5 milliamperes per gel column. The relative amount of each isoenzyme present was quantitated by densitometric scanning (ERI 10, Carl Zeiss Jena, Germany). Activity bands were visualized using lactate and NAD as substrates and nitro blue tetrazolium as final hydrogen acceptor. The LD isoenzyme with the highest electrophoretic mobility towards the anode was designated LD-1 and that with the lowest mobility LD-5 (Makeawa, 1988).

Total LD and CK enzyme activities are given in katals. The LD isoenzymes are quantified as a percentage of total LD enzyme present in the serum.

Group means, standard deviations, standard errors and coefficients of variation were established for each factor. The statistical significance of differences between groups of animals was evaluated by Student's *t*-test. Differences of $P < 0.01$ were regarded as significant.

Results

Table 1 presents serum creatine kinase (CK) activities in two groups of piglets (healthy and affected) and in their parents. The mean value of CK activity for 6 adult animals in the tested groups was 19.57 ± 3.56 μkat · l^{-1}1, and the mean values of CK activity for all piglets in groups 1 and 2 were $21.03 \pm$ ± 1.33 μkat · l^{-1} and 20.42 ± 1.23 μkat · l^{-1}, respectively. Significant differences in CK activity were demonstrable neither between the healthy and the affected groups nor between males and females. The mean serum CK levels found by us for pigs and piglets were different from the reference values of serum CK reported by various authors (see Table 2).

Values (absolute and per cent activity) of total LD enzyme and LD isoenzyme activities found in the serum of both groups of piglets and their parents are shown in Tables 3 to 5. In piglets of groups 1 and 2, total LD activity of the serum ranged from 13.16 ± 0.37 μkat · l^{-1} to 14.24 ± 0.09 μkat · l^{-1}

Table 1

Serum CK activity (μkat·l^{-1}) in two groups of Prestice black pied pigs

			Animal (Line)	Sex	Range (min—max)	Mean ± SD	SEM	CV
Group 1	Family 1	Parents	Jura — 51 (1430)	M	21.97–22.26	22.14±0.136	0.06	0.61
			Adamosa — 11722	F	18.86–19.32	19.09±0.189	0.08	0.99
		Litter	Piglet 1	F	18.87–20.45	19.50±0.736	0.33	3.77
			Piglet 2	F	22.37–23.45	22.99±0.499	0.22	2.17
			Piglet 3	M	20.93–22.34	21.64±0.560	0.25	2.59
	Family 2	Parents	Sapon — 3001 (559)	M	24.73–24.95	24.86±0.093	0.04	0.37
			Jurka 11623	F	16.99–17.41	17.23±0.170	0.08	0.99
		Litter	Piglet 4	M	19.78–21.12	20.49±0.563	0.25	2.75
			Piglet 5	F	19.85–21.56	20.52±0.738	0.33	3.60
Group 2		Parents	Piratek — 72 (559)	M	19.23–19.45	19.33±0.086	0.04	0.44
			Sow — 17288	F	14.58–14.89	14.78±0.123	0.06	0.83
		Litter	Piglet 6	F	17.83–20.12	19.03±1.064	0.48	5.59
			Piglet 7	F	20.98–21.76	21.35±0.342	0.15	1.60
			Piglet 8	M	20.22–21.47	20.89±0.552	0.25	2.64

CV = coefficient of variation; SD = standard deviation; SEM = standard error of the mean; M = male; F = female

Table 2

Reference values for serum CK activity (μkat·l^{-1}) in adult pigs

Reference	Mean ± SD	Range	Note
The Merck Veterinary Manual, Sixth Edition (1986)	4.634±3.538	1.097–8.172	Valid for all pigs
Vrzgula — Sokol (1987)	0.885±0.215	0.67–1.1	Valid for all pigs
Reese et al. (1984)	6.407±4.149	1.52–16.58	Gilts, gestation day 110
	5.814		Sows, gestation day 110
	4.340		Sows, lactation day 14
	3.487		Sows, lactation day 26
Rozkošná et al. (1986)	11.52±3.8	4.8 –18.2	Belgian Landrace breed
	2.68±0.99	1.45– 4.45	Large White Breed
	2.11±0.66	1.40– 3.50	Laboratory minipigs

Table 3

Serum LD activity (μkat·l^{-1}) in two groups of Prestice black pied pigs

			Animal (Line)	Sex	Range (min—max)	Mean ± SD	SEM	CV
Group 1	Family 1	Parents	Jura — 51 (1430)	M	5.549–6.021	5.78±0.094	0.04	1.63
			Adamosa — 11722	F	9.532–9.846	9.68±0.053	0.02	0.55
		Litter	Piglet 1	F	12.759–13.681	13.16±0.370	0.17	2.81
			Piglet 2	F	14.001–14.531	14.24±0.090	0.04	0.63
			Piglet 3	M	13.254–13.725	13.56±0.080	0.04	0.59
	Family 2	Parents	Sapon — 3001 (559)	M	6.491–8.324	6.96±0.340	0.15	4.89
			Jurka 11623	F	8.225–8.688	8.46±0.080	0.04	0.95
		Litter	Piglet 4	M	13.243–13.831	13.64±0.110	0.05	0.81
			Piglet 5	F	13.907–14.253	14.08±0.070	0.03	0.50
Group 2		Parents	Piratek — 72 (559)	M	4.642–5.354	5.001±0.13	0.06	2.6
			Sow — 17288	F	9.636–9.840	9.79±0.040	0.02	0.41
		Litter	Piglet 6	F	10.103–10.980	10.50±0.180	0.08	1.71
			Piglet 7	F	8.325–8.982	8.98±0.090	0.04	1.00
			Piglet 8	M	8.813–9.541	9.12±0.120	0.05	1.32

See footnote to Table 1

and from 8.98 ± 0.09 μkat · l^{-1} to 10.50 ± 0.18 μkat · l^{-1}, respectively, and the difference reached the level of significance ($P < 0.01$).

Elevations in LD-5 isoenzyme activity were noted in piglets affected with congenital tremor. At the same time, these piglets demonstrated a significant ($P < 0.01$) decrease in LD-2 isoenzyme activity as compared to the control group. No significant differences in total LD and LD isoenzyme activities were found between sows and boars of affected ($n = 4$) and nonaffected ($n = 2$) groups of piglets. However, total LD activity and LD-5 isoenzyme activity was significantly ($P < 0.01$) higher while LD-2 isoenzyme activity was significantly ($P < 0.01$) lower in sows.

The reference values reported by various authors for total serum LD and LD isoenzyme activity are presented in Tables 4 and 6.

Table 4

Reference values for serum LD activity in pigs ($\mu kat \cdot l^{-1}$) according to different authors

Reference (year)	Mean ± SD	Range	Note
The Merck Veterinary Manual, Sixth Edition (1986)	4.88±3.13	2.67–7.09	Valid for all pigs
Vademecum (Sokol, 1991)	<10.00		Valid for all pigs
Akita et al. (1980)	9.59±0.42	9.17–9.96	Japanese Landrace pigs
Reese et al. (1984)	2.41±0.61	1.052–3.79	Gilts, gestation day 110
	2.378		Sows, gestation day 110
	2.53		Sows, lactation day 14
	2.68		Sows, lactation day 28
Tuček et al. (1985)	10.132±0.926		Piglets, 8 hours after birth
	7.114±0.352		Piglets, 21 days after birth
Doize et al. (1989)		5.28 –20.42	Adult Landrace pigs
Szilágyi et al. (1989)	10.59±4.025		9-week-old Landrace piglets
	14.66±4.325		7-week-old Landrace piglets
	6.11		Adult crossbred pigs

Discussion

Serum enzymes like creatine kinase and lactate dehydrogenase are widely used as indicators of myopathies in animals as well as in humans (DiBartola and Tasker, 1977; Maekawa, 1988; Kursa and Kroupova, 1982; Roodenburg et al., 1990; Tuček et al., 1985). In healthy individuals, the concentration of such enzymes in the serum is low. It may, however, rise by one or several orders of magnitude due to an increase in cell membrane permeability, e.g. after muscle damage (Duthie et al., 1989; Gupta et al., 1991; Roodenburg et al., 1990; Tuček et al., 1985). It therefore appeared to be of interest to elucidate the changes occurring in the profile of these enzymes in our test material derived from a congenital tremor affected pig herd.

The diagnostic usefulness of serum CK and LD enzyme testing in myopathies lies primarily in their high (0.57–0.86) coefficient of repeatability (Mojto et al., 1981). The reference values reported by different authors for the two

Table 5

LD isoenzyme activities, expressed as a percentage of total LD present in the serum, in two groups of Prestice black pied pigs

		Animal (line)	Sex	LD isoenzyme activity ($\bar{x} \pm Sx$)				
				LD-1	LD-2	LD-3	LD-4	LD-5
Group 1	Family 1 Parents	Jura 51 (1430)	M	43.40±0.26	18.24±0.25	18.60±0.11	6.46±0.17	13.30±0.13
		Adamosa — 11722	F	39.80±0.61	11.20±0.40	17.02±0.32	13.34±0.33	18.64±0.71
	Family 1 Litter	Piglet 1	F	41.62±0.57	11.26±0.29	18.72±0.38	6.30±0.31	22.10±0.39
		Piglet 2	F	40.46±0.47	12.74±0.27	17.46±0.20	7.08±0.12	22.26±0.39
		Piglet 3	M	43.58±0.22	11.30±0.30	17.00±0.16	8.78±0.18	13.34±0.66
	Family 2 Parents	Sapon 3001 (559)	M	43.90±0.21	17.56±0.21	18.68±0.16	6.86±0.13	12.96±0.35
		Jurka — 11623	F	41.24±0.29	10.74±0.25	18.30±0.23	12.76±0.21	16.96±0.42
	Family 2 Litter	Piglet 4	M	42.34±0.29	12.28±0.14	19.10±0.20	6.24±0.23	20.04±0.45
		Piglet 5	F	45.06±0.28	12.10±0.17	17.16±0.21	7.18±0.10	18.50±0.28
Group 2	Parents	Piratek 72 (559)	M	44.56±0.32	17.44±0.13	18.50±0.25	8.30±0.18	11.20±0.52
		Sow — 17288	F	45.22±0.39	12.14±0.42	15.34±0.24	14.20±0.27	13.30±0.43
	Litter	Piglet 6	F	44.68±0.29	19.02±0.24	19.06±0.17	7.96±0.24	9.28±0.32
		Piglet 7	F	44.28±0.26	18.66±0.22	18.44±0.25	8.48±0.15	10.14±0.32
		Piglet 8	M	44.50±0.17	19.38±0.09	18.96±0.09	9.04±0.10	8.12±0.17

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Table 6

Reference values for serum LD isoenzyme activities in pigs (% of total LD activity) according to different authors

Reference (year)	LD-1	LD-2	LD-3	LD-4	LD-5	Note
Barthel et al. (1971)	30.6	17.5	16.9	14.3	20.7	Young pigs (14–28 kg)
	20.3	15.4	19.7	18.7	19.9	Slaughter group (85–110 kg)
Doize et al. (1989)	17	15	18	10	40	Landrace pigs (70–89 kg)
Michalek and Marcaník (1975)	44.4 (39–52)	24.9 (19–26)	13.6 (9–15)	8.8 (4–12)	8.3 (3–12)	Slovak Large White (over 60 kg)

enzymes (Tables 2 and 4) are dissimilar, which may be due to variations in the methods of assay by laboratory.

Serum CK activity is used in the diagnosis of muscular diseases because of its high tissue specificity (Bickhardt, 1983). The mean CK levels found for pigs and piglets in this study were significantly ($P < 0.01$) higher than those reported previously (Table 2). Mean CK value determination was not used in piglets, since CK activity shows large variations in young animals (Bickhardt, 1983; Rozkosna et al., 1986). The difference between CK values found in the present study and those reported in the literature may be due to several factors such as (1) breed (Doize et al., 1990; Lukas et al., 1978; O'Brien et al., 1985; Rozkosna et al., 1986; Szilágyi et al., 1982; Toulova et al., 1981), (2) sex (Jakubowski et al., 1988), (3) age (Heffron and Mitchell, 1975), (4) nutrition (Duthie et al., 1989; Toulova et al., 1981), (5) reproductive status and stage of lactation (Reese et al., 1984).

The genetic predisposition of animals to stress was also demonstrated to enhance serum CK activity (Poltarsky et al., 1989). In stress-susceptible animals (Akita et al., 1980; Bickhardt et al., 1977; Borgman et al., 1985; Doize et al., 1990), CK levels undergo a multiple increase during the first hour after the stress (Hallberg et al., 1979). According to other authors, the following stress factors could account for the increased CK activity in this study: handling and manipulation of animals (Doize et al., 1989; Toulova et al., 1981), venipuncture (King and Zapf, 1972; Sano et al., 1979) and intramuscular injection (Steiss and Forsyth, 1984) of vitamin E (Duthie et al., 1989; Toulova et al., 1981).

While CK activity is mainly present in skeletal muscle, the activity of LD reflects no damage to a particular tissue (Barthel et al., 1971; Gupta et al., 1991; Maekawa, 1988). Therefore, additional information may be obtained through the separation and quantitation of five electrophoretically distinct LD isoenzymes (Akita et al., 1980; Barthel et al., 1971; Doize et al., 1989; Maekawa, 1988; Michalek and Marcanik, 1975; Rozkosna et al., 1986).

An increase in total LD activity above the upper limit of reference values (Table 4), similar to that seen in this study, may be evoked by gestation and lactation in sows (Reese et al., 1984) and by muscle cramps, trembling and muscle fibre cytoplasmic membrane damage (Doize et al., 1990; Duthie et al., 1989; Gupta et al., 1991; Roodenburg et al., 1990) in piglets affected with congenital tremor.

The LD isoenzyme pattern found in this study largely agrees with data obtained for pigs by Barthel et al. (1971), Doize et al. (1989) and Michalek and Marcanik (1975). We have not observed age-related changes in LD isoenzyme distribution as reported by Barthel et al. (1971). As compared to the reference values (Table 5), changes LD-4 and LD-5 isoenzyme activity in sows apparently reflect normal alterations in metabolic processes concurrent with

delivery and the onset of lactation (Wright and Grammer, 1980). On the other hand, the higher levels of LD-5 isoenzyme could be due to a genetically encoded stress susceptibility of the sows tested (Akita et al., 1980; Rozkosna et al., 1986).

The significantly ($P < 0.01$) elevated LD-5 isoenzyme values obtained for piglets affected with congenital tremor may be attributed to muscle tissue damage (Akita et al., 1980).

It can be concluded that no significant correlation was demonstrated between CK activity and the incidence of congenital tremor in piglets. As serum CK values were similar for both the affected and the unaffected group, we assume that the measurement of CK activity is not useful in the diagnosis of congenital tremor. At the same time, the positive relationship found between congenital tremor and elevated LD enzyme and LD-5 isoenzyme activities allows us to suggest that total LD and LD-5 isoenzyme activity may be used as a biological marker of congenital tremor in piglets.

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IS THERE LIPID PEROXIDATION INDUCED MALONDIALDEHYDE PRODUCTION DURING EGG SHELL FORMATION?

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The marked increase observed in malondialdehyde concentration of the blood plasma and liver of laying hens during egg shell formation in a previous experiment (Mézes and Lencsés, 1985) were explained as a possible consequence of physiologically controlled lipid peroxidation. In this experiment, 54-week-old laying hens were treated intrauterinally with indomethacin (1.0 and 2.0 mg/kg body mass) after oviposition. Two other groups of hens were treated *per os* with excess amounts of vitamin E (100 and 200 mg/bird). The higher dose of indomethacin significantly decreased the $PGF_{2\alpha}$ production and malondialdehyde content of the sell gland, as well as the malondialdehyde concentration of the plasma. The excess amount of vitamin E had the same effect. The results suggest that during egg shell formation malondialdehyde is derived from prostanoid biosynthesis rather than from a free-radical initiated lipid peroxidative process. On the other hand, the excess amount of vitamin E inhibited prostaglandin biosynthesis as well as malondialdehyde production.

Key words: Malondialdehyde, prostaglandins, vitamin E, laying hen, egg shell formation

Sexual endocrinological processes of the laying hen have some specific properties. Anatomically, egg shell formation takes place in the uterus or shell gland (Gilbert and Pearson, 1982). Prostaglandins are synthesized in the ovary and uterus within the reproductive tract (Wechsung, 1983; Hester et al., 1991). They have an important role in the contraction of the oviduct and uterus (Wechsung and Houvenaghel, 1978, 1980). Indomethacin, as an inhibitor of prostaglandin biosynthesis, was found to delay oviposition (Balog and Hester, 1991*a*) and had an effect also on egg-shell quality (Balog and Hester, 1991*b*). Among other molecules, cyclic endoperoxides are also formed during prostaglandin biosynthesis. One of the end-products of cyclic endoperoxide formation is malondialdehyde (Gorman, 1978). Malondialdehyde was also found to be a good marker of uncontrolled lipid peroxidative processes (Gutteridge and Quinlan, 1983). Malondialdehyde concentration of the blood plasma and liver was reported to increase markedly during egg shell formation (Mézes and Lencsés, 1985) when it was proposed to be an end-product of physiologically controlled lipid peroxidation.

The aim of this study was to shed light on the above-mentioned problem by inhibiting prostanoid biosynthesis with the generally accepted cyclooxy-

genase inhibitor indomethacin (Shimada and Asai, 1979), and by inhibiting lipid peroxidative processes using the naturally occurring chain-breaking antioxidant, vitamin E (Panganamala and Cornwell, 1982).

Materials and methods

The experimental birds were 54-week-old laying hens of the Rhode breed. Indomethacin treatment was carried out intrauterinally with doses of 1.00 and 2.00 mg/kg body mass in a volume of 0.5 ml, dissolved in propylene glycol (Elmetacin, Luitpold GmbH, Munich). The original solvent of indomethacin was 70% (v/v) i-propanol. For this reason, a solvent control group was treated with 0.5 ml propylene glycol containing 0.1 ml propanol. The birds' reproductive status was examined by palpation through the cloaca: all birds showed post-oviposition status. Samples were taken after cervical dislocation approximately 6 h following the treatment. Vitamin E treatment was performed orally, with enterosolvent capsules (Vitamin E capsule, Chemical Works of G. Richter Ltd., Budapest) containing 100 or 200 mg vitamin E per kg body mass. The hens' reproductive status was the same as mentioned above. Samples were taken approximately 24 h after treatment.

Prostaglandin $F_{2\alpha}$ content of the uterus was measured after homogenizing the organ in a fivefold volume of a 3 : 1 (v/v) mixture of acetonitrile and ethanol, in a Potter–Elvehjem teflon homogenizer, and with extraction of the same solvent at 60 °C for 10 min. An aliquot of the extract was evaporated in vacuum chamber at 60 °C and –8 att by the method of Bálint (personal communication). Recovery was determined by the addition of unlabelled $PGF_{2\alpha}$ and was found to be 64.5%. $PGF_{2\alpha}$ was quantified by ^{125}I radioimmunoassay (Institute of Isotopes, Hungarian Academy of Sciences, Budapest). Malondialdehyde content of the whole uterus homogenate was determined by the method of Mihara et al. (1980) and that of the blood plasma was measured according to Placer et al. (1966). Vitamin E content was measured in the uterus as described by Haacker (1976) and in the blood plasma as recommended by Bieri (1964).

Statistical analysis of the results was performed by paired Student's *t*-test (Snedecor and Cochran, 1976).

Results

Prostaglandin $F_{2\alpha}$ content of the uterus decreased significantly in the groups treated with the higher dose of indomethacin (2.0 mg/kg b.m.) and also after treatment with vitamin E at a dose of 200 mg/bird (Table 1). The lower

Table 1

Effect of indomethacin and vitamin E treatment on prostaglandin and malondialdehyde production of the shell gland of laying hens (mean ± SD)

Group/treatment	$PGF_{2\alpha}$ (pg/g)	Malondialdehyde (nmol/g)	Vitamin E (mg/g)
1. Control	538.55	3.09	0.096
(n = 5)	102.99	0.63	0.021
2. Solvent-treated	527.58	2.17	0.097
control (n = 5)	147.99	0.97	0.040
3. Indomethacin	405.95	2.07	0.085
1.0 mg/kg (n = 5)	74.80	0.27	0.022
4. Indomethacin	267.39	1.82	0.087
2.0 mg/kg (n = 5)	85.54	0.42	0.040
5. Vitamin E	693.24	2.21	0.097
100 mg/hen (n = 5)	179.61	0.46	0.006
6. Vitamin E	344.05	2.06	0.107
200 mg/hen (n = 5)	135.55	0.14	0.012
Levels of significance:			
1 vs 2	n. s.	n. s.	n. s.
2 vs 3	n. s.	n. s.	n. s.
2 vs 4	$P < 0.05$	$P < 0.01$	n. s.
1 vs 5	n. s.	n. s.	n. s.
1 vs 6	$P < 0.05$	$P < 0.01$	n. s.

dose of indomethacin (1.0 mg/kg b. m.) caused a non-significant decrease, while the lower dose of vitamin E (100 mg/bird) a non-significant increase, in uterine prostaglandin production (Table 1).

Malondialdehyde content of the uterus decreased significantly in hens treated with the higher dose of indomethacin as well as in those receiving the higher dose of vitamin E (Table 1). Neither indomethacin nor vitamin E treatment caused significant changes in the vitamin E content of the uterus (Table 1).

Malondialdehyde concentration of the blood plasma decreased significantly in all treated groups but the solvent-treated control (Table 2). Plasma vitamin E concentration showed no significant changes (Table 2).

Discussion

Both the cyclooxygenase inhibitor indomethacin and the natural chain-breaking antioxidant vitamin E were found to reduce the rate of prostanoid biosynthesis in the hens' uterus. These effects of indomethacin are well known. The results obtained in the present study for vitamin E support some previous *in vitro* and *in vivo* findings concerning the inhibitory effect of vitamin E on the arachidonic acid cascade mechanism (Panganamala and Cornwell, 1982).

Table 2

Effect of indomethacin and vitamin E treatment on blood plasma malondialdehyde and vitamin E concentration (mean ± SD)

Group/treatment	Malondialdehyde (nmol/ml)	Vitamin E (mg/l)
1. Control	3.94	1.94
(n = 5)	1.30	0.31
2. Solvent-treated	4.04	1.84
control (n = 5)	1.04	0.51
3. Indomethacin	2.85	1.97
1.0 mg/kg (n = 5)	0.51	0.65
4. Indomethacin	2.44	1.79
2.0 mg/kg (n = 5)	0.32	0.56
5. Vitamin E	2.49	2.02
100 mg/hen (n = 5)	0.18	0.21
6. Vitamin E	1.90	2.16
200 mg/hen (n = 5)	0.18	0.91
Levels of significance:		
1 vs 2	n. s.	n. s.
2 vs 3	$P < 0.05$	n. s.
2 vs 4	$P < 0.05$	n. s.
1 vs 5	$P < 0.05$	n. s.
1 vs 6	$P < 0.01$	n. s.

The results of this study also suggest that an excess amount of dietary vitamin E may alter egg formation and oviposition through its effect exerted on prostaglandin biosynthesis.

The malondialdehyde content of the uterus and the blood plasma was decreased by both indomethacin and/or vitamin E treatment. This finding does not support our previous hypothesis concerning a physiologically controlled lipid peroxidation during egg shell formation (Mézes and Lencsés, 1985) but suggests an alternative mechanism. Malondialdehyde probably does not originate from a free-radical generated process; rather, it is an end-product of prostaglandin biosynthesis. If it were a product of lipid peroxidation, only vitamin E, but not indomethacin, treatment would cause a decrease, as indomethacin does not act as an antioxidant (or scavenger). The results also direct attention towards the importance of proper vitamin E supplementation, as — given at levels in excess of the requirement — that biologically active molecule may alter the processes of egg shell formation and oviposition.

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MORPHOLOGICAL STRUCTURE OF THE MUCOUS MEMBRANE AND SUBMUCOSA OF RUMEN IN CALVES RECEIVING SYNTHETIC OR NATURAL β-CAROTENE AND VITAMINS AD_3E*

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In an experiment conducted on 20 calves from 1 day to 12 weeks old, supplements of β-carotene were fed in the form of Rovimix β-carotene 10%, artificially dehydrated carrot or vitamins AD_3E. Postmortem examination carried out at 12 weeks showed that supplementation of β-carotene or vitamins AD_3E resulted in a better structural development of the ruminal papillae as compared to the control group. In addition, β-carotene reduced the keratinization of the stratified squamous epithelial cell layer of the rumen and increased the glycosaminoglycane level of that organ wall.

Key words: β-carotene, vitamins AD_3E, supplementation, calf, rumen, mucous membrane, morphological structure

Postnatal development of the alimentary tract greatly depends on the type and composition of feeds fed to preruminant calves. A properly balanced daily ration is essential not only for a better development of the digestive tract but also for the highest possible utilization of all nutrients contained in the ration. Different biologically active compounds, such as vitamins and microelements, have a well-known role in the optimum utilization of nutrients. Moreover, a deficiency of these compounds renders animals susceptible to different diseases (e.g. symptomatic diarrhoea and pneumonia) and may lead to death (Roliński, 1985; Winter, 1985; Flachowsky et al., 1990), particularly during the first few weeks of life (Łuczak, 1978; Łuczak and Depta, 1984; Ray, 1988; Sowers and Wallace, 1990; Tioelker et al., 1990).

Vitamin A and its precursor β-carotene belong to such essential active compounds. The transplacental transport of vitamin A from mother to fetus is known to be relatively poor (Branstetter et al., 1973; Bárdos et al., 1991). Thus, newborn calves suffer from β-carotene deficiency (Rzedziecki and Chmielewski, 1976). To avoid this deficiency, it is recommended to supplement the calf ration with β-carotene and vitamin A (Falkowska and Iwańska, 1978; Iwańska et al., 1986).

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The influence of these compounds on postnatal structural development of the wall of the digestive tract, particularly the rumen, is not known. The objective of this study was, therefore, to monitor the influence of natural and synthetic β-carotene as well as vitamin A on the morphological structure of the rumen wall.

Materials and methods

The experiments were conducted on 20 male calves of the Black-and-White Lowland breed, from birth up to 12 weeks of age. The calves were born from December to February by multiparous cows. The animals were kept in individual pens and fed according to the standard norms of feeding and housing. The calves were fed colostrum and whole milk up to 3 weeks of age. Subsequently, they were shifted to standard quantities of milk substitute and concentrate mix, and hay given *ad libitum*. Up to 7 weeks of age, the calves were given 0.8 kg milk substitute, between 8 and 10 weeks of age they received 0.6 kg, and thereafter up to 11 weeks 0.4 kg milk substitute per animal daily. The milk substitute was given twice and the concentrate mix once daily. The chemical composition of the feeds is presented in Table 1.

The calves were randomly divided into 4 groups receiving β-carotene and vitamin A from different sources (groups 2—5) and a control group (group 1) without any supplementation (the experimental protocol is shown in Table 2).

The feed of animals in groups 2 and 3 was supplemented with Rovimix β-carotene (Hoffman–La Roche) containing 100 mg of synthetic β-carotene per gram in a stabilizing matrix of gelatin, dehydrated and supplied with antioxidants. Group 4 was given artificially dehydrated carrots which contained 0.920 mg β-carotene per gram. These supplements were given once daily, mixed in liquid feed.

Animals of all groups were slaughtered at 12 weeks of age. Postmortem examination of the calves, with special regard to the rumen, was performed immediately after slaughtering. At the same time, five pieces from different parts of the rumen wall (the dorsal, ventral and cranial sacs and the caudo-

Table 1

Chemical composition of feeds (in %)

Feed	Dry matter	Ash	Crude	Ether extract	Crude fibre	N-free extract
Milk substitute	92.62	8.16	28.20	11.58	0	44.68
Mixture CJ	86.74	6.24	18.98	3.41	4.56	53.55
Meadow hay	87.45	7.06	9.52	1.21	29.79	39.87

Table 2

Design of the experiment

Group	Number of calves	Type of supplements	Doses of supplements for calves		
			period of treatment	daily doses	
			in weeks	β-carotene (mg)	vit. AD_3E (g)
1	4	without supplements			
2	4	Rovimix β-carotene 10%	0–1	5	1
3	4	Rovimix β-carotene 10% vitamins AD_3E	2–3 4	10 15	2 2
4	4	artificially dehydrated carrot	5 6–9	20 25	2 3
5	4	vitamins AD_3E	10–13	30	3

1 g vitamins AD_3E: 1500 IU of vit. A
750 IU of vit. D_3
10 IU of vit. E

Table 3

The effect of supplementations with natural or synthetic β-carotene and vitamins AD_3E on the histochemically determined level of glycosaminoglycanes in the rumen wall

Group	Type of supplements	Level of glycosaminoglycanes in ruminal wall*	
		from	to
1	without supplements (control group)	+	++
2	Rovimix β-carotene 10%	+++	++++
3	Rovimix β-carotene 10% vitamins AD_3E	+++	++++
4	artificially dehydrated carrot	++	++++
5	vitamins AD_3E	++	++++

*Intensity of colour reaction:	Concluded level of glycosaminoglycanes:
+ weak	low
++ slightly stronger	slightly higher
+++ strong	high
++++ very strong	very high

dorsal as well as caudoventral blind sacs) were collected from each calf and fixed in neutral formalin for microscopic examination. Paraffin sections were stained with haematoxylin and eosin. In order to determine the behaviour of glycosaminoglycanes in the rumen wall, the sections were stained by the PAS reaction according to McManus.

The following criteria were adopted for the microscopic evaluation of the ruminal sections: (1) degree of development of the papillae (weak, satisfactory, good, very good); (2) structure of the papillae and of the stratified squamous epithelium (loose, slightly loose, compact); (3) degree of keratinization of the epithelium (weak, satisfactory, substantial); (4) pattern of the keratin layers (compact, loose, slightly loose, fluffy, clearly visible cells).

The level of glycosaminoglycanes was estimated by the intensity of the histochemical colour reaction as described by Szarek (1984). The control consisted of digestion with diastase (Pearse, 1968).

Results

Macroscopically, the organs and tissues of all calves showed normal morphological structure. The histological pattern of the mucous membrane and submucosa of the rumen in the different groups is described below.

Group 1 (control, given no supplement). Well-developed papillae were seen in all but one calf which showed a low degree of papillary development. The structure of these papillae was slightly loose, and one calf had a predominantly loose structure. The stratified squamous epithelial layers of the rumen wall had loose composition in many sections (Fig. 1A). In one calf, some areas of the ruminal mucous membrane showed on overhanging fluffy layer of epithelium (Fig. 1B). A substantial degree of keratinization was almost consistently present, but in only few cases did it reach a satisfactory level.

Group 2 (ration supplemented with 10% Rovimix β-carotene). The degree of development of the ruminal papillae of group 2 calves could be described as good and very good. Their structure was slightly loose, with a greater proportion being transformed into compact. The stratified squamous epithelial layer was slightly loose and in some cases it became compact (Fig. 2A). Keratinization of the epithelium was of satisfactory degree and in a few cases it was slightly more developed. Parts of the keratin layer were often insufficiently developed and occasionally varied from loose to compact. A greater proportion of the keratin layer had well-visible epithelial cells present in its structure (shown with asterisks in Fig. 2A).

Group 3 (ration supplemented with 10% Rovimix β-carotene and vitamins AD_3E). The ruminal papillae of group 3 calves were very well developed with a somewhat loose structure, and the epithelium was often slightly loose (Fig. 3).

The stratified squamous epithelial layer was satisfactory developed, but in some cases it showed a lower degree of development. The keratin layer was usually compact and contained, in addition to necrotic cells, living cells as well (as indicated with asterisks in Fig. 3).

Group 4 (ration supplemented with dehydrated carrot). Calves of group 4 had predominantly well-formed papillae. In one case, the papillae were very well developed, and in another case their development was satisfactory. The structure of all papillae as well as of the epithelium was distinctly compact in about 75% of the sections, and in these cases epithelial keratinization was weaker. In other sections, the papillae had a slightly looser structure, epithelization was satisfactory, and the superficial epithelial layer was mostly compact or slightly loose (Fig. 4).

Group 5 (ration supplemented with vitamins AD_3E). The ruminal papillae of group 5 calves were well or very well developed, and the tissue was compact or slightly loose. The stratified squamous epithelium was rather compact (Fig. 5A) and sometimes tended to be loose. The degree of epithelial keratinization could be described as satisfactory. In some places there was slight desquamation. A greater proportion of the compact keratin layer contained epithelial cells of distinct structure (Fig. 5B).

The histochemical reaction of glycosaminoglycanes present in the mucous membrane and submucosa of the rumen in calves of groups 1, 2 and 4 is shown in Figs 1C, 2B, 4B and 4C. The level of that substance in the organs studied is presented in Table 3.

Discussion

In this study, differences were demonstrated in the morphology of the ruminal mucosa and submucosa of 12-week-old calves, depending on whether their ration was supplemented with synthetic or natural β-carotene and vitamin AD_3E. The ruminal papillae were most developed in the calves which received Rovimix β-carotene 10% with or without vitamin AD_3E. A lesser degree of papillary development was observed in animals given a meal of dehydrated red carrots or vitamin AD_3E alone. The weakest development of papillae was noted in the control group. This fact is supported by the observation that oral administration of β-carotene to calves resulted in elevated levels of β-carotene and vitamin A in the blood serum (Iwańska et al., 1988). It can be concluded that the development of ruminal papillae observed in this study was due to the effect of vitamin A, a vitamin having a definite role in the development of epithelial and connective tissue (Roliński, 1985; Rzedziecki and Chmielewski, 1976).

Besides the above-mentioned factors, the presence of solid component in the feed also influenced the development of ruminal papillae. It is known that

calves in the early stage of their life do not show much interest in solid feed (Curnick et al., 1983). At the same time, Pysera et al. (in press) found that the administration of synthetic or natural β-carotene to calves brought about a twofold increase in the intake of solid feed (meadow hay and mixture CJ). It was also reported that the inclusion of large amounts of solid feed in calf rations not only stimulated ruminal movements but also had beneficial effects on the development of the mucous membrane of that organ (Zduńczyk and Lewicki, 1985; Szarek et al., in press).

In this study, animals not receiving β-carotene or vitamins showed differences in the degree of keratinization of the stratified squamous epithelial layer as compared to the other groups. However, animals in the other groups also showed a low degree of keratinization and desquamation of epithelial cells.

The keratin layer of the epithelium was the loosest in the control animals, while in other calves it was more compact. Animals receiving 10% β-carotene, alone or with vitamins AD_3E, had more cells of distinct structure in the keratin layer. The above differences also appeared to have resulted from the role of epithelial cells in retinol metabolism. Excessive desquamation of epithelial cells, observed in the control animals, might have resulted from a deficiency of vitamin A, a condition frequently observed in calves during the first week of life (Roliński, 1985). As regards the level of glycosaminoglycanes in the ruminal mucosa and submucosa, it was lower in the control animals and higher in calves given β-carotene 10% and vitamins AD_3E. Such localization of glycosaminoglycanes is indicative of better ruminal function and results in a better digestion of organic substances and crude fibre when the animals are given β-carotene.

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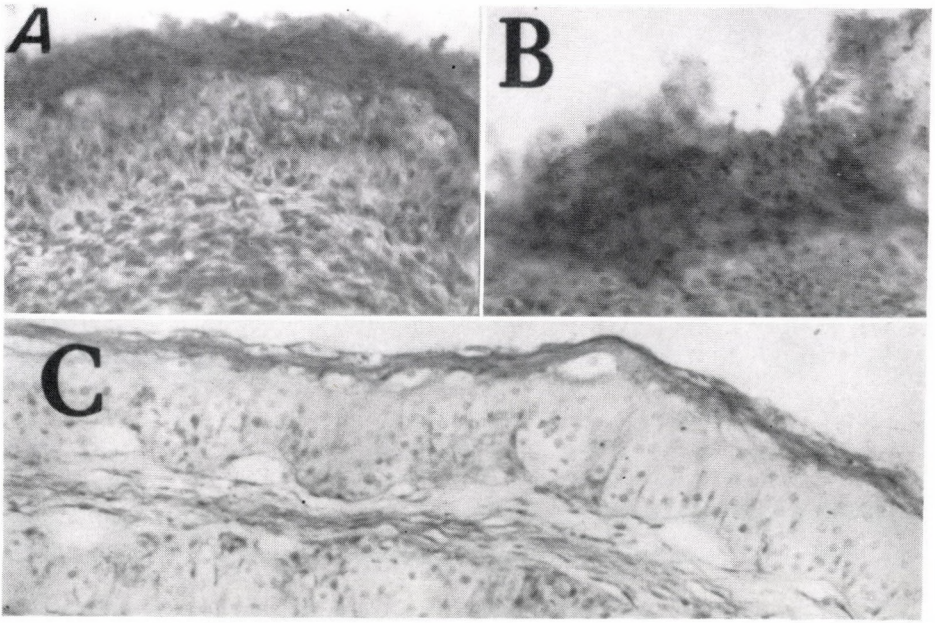

Fig. 1. Rumens from the control calves. (A) Loose structure of the stratified squamous epithelium with fluffy keratin layer. (B) The keratin layer of epithelium is seen in the form of fluffy overhangings. Haematoxylin and eosin (H.-E.), ×240. (C) Weak reaction for glycosaminoglycanes. PAS reaction according to McManus, ×240

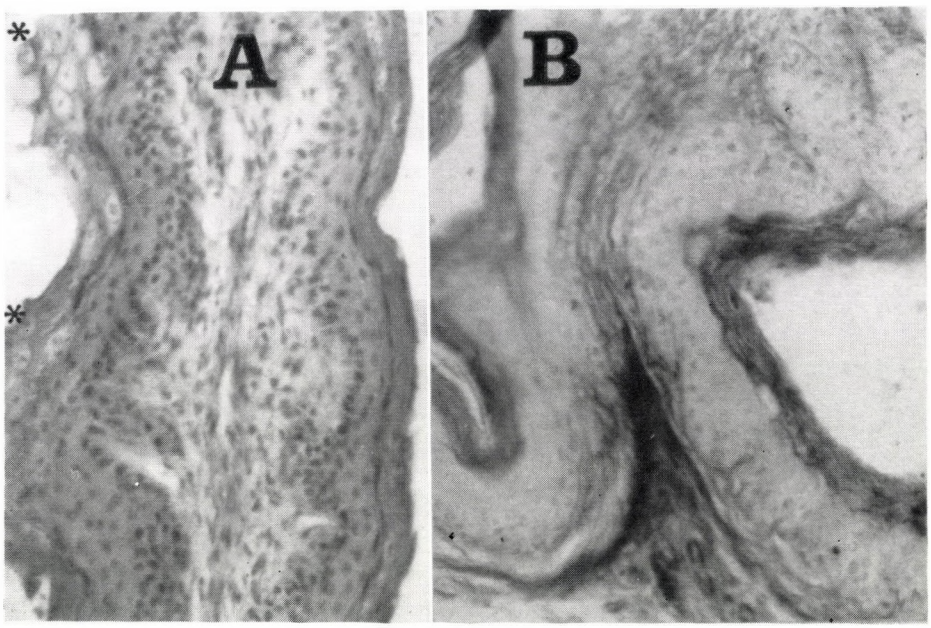

Fig. 2. Rumens from group 2 calves. (A) Slightly loose structure of the stratified squamous epithelium with loose keratin layer and clearly visible cells (asterisks). H.-E., ×240. (B) Strong — in places very strong — reaction for glycosaminoglycanes. PAS reaction according to McManus, ×240

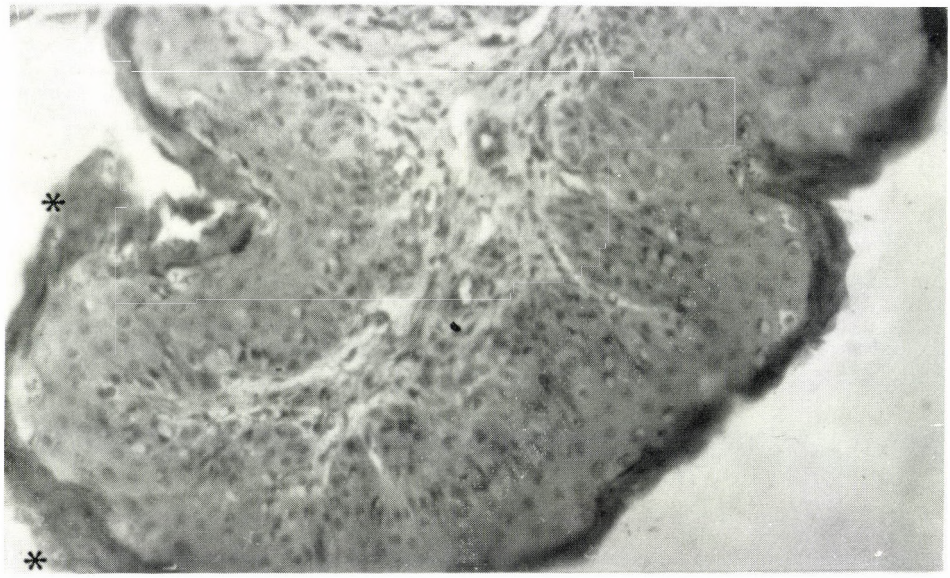

Fig. 3. Rumen from group 3 calf. Well-developed papillae with slightly loose stratified squamous epithelium having a compact keratin layer and areas of clearly visible cells (asterisks). H.-E., ×240

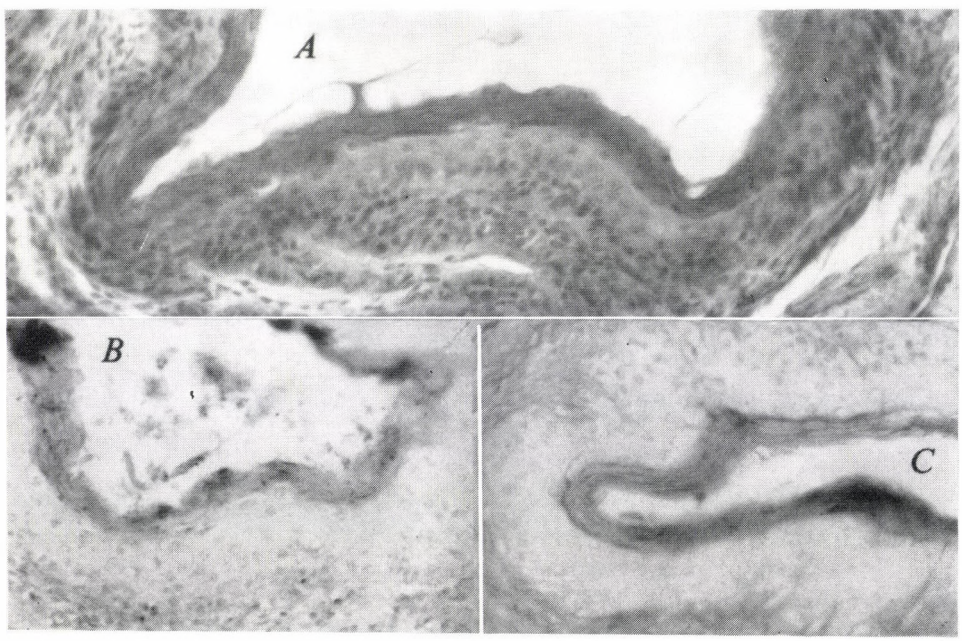

Fig. 4. Rumens from group 4 calves. (A) Slightly loose structure of the stratified squamous epithelium. H.-E., ×240. (B, C) Differential reaction for glycosaminoglycanes (from moderate to very strong). PAS reaction according to McManus, ×240

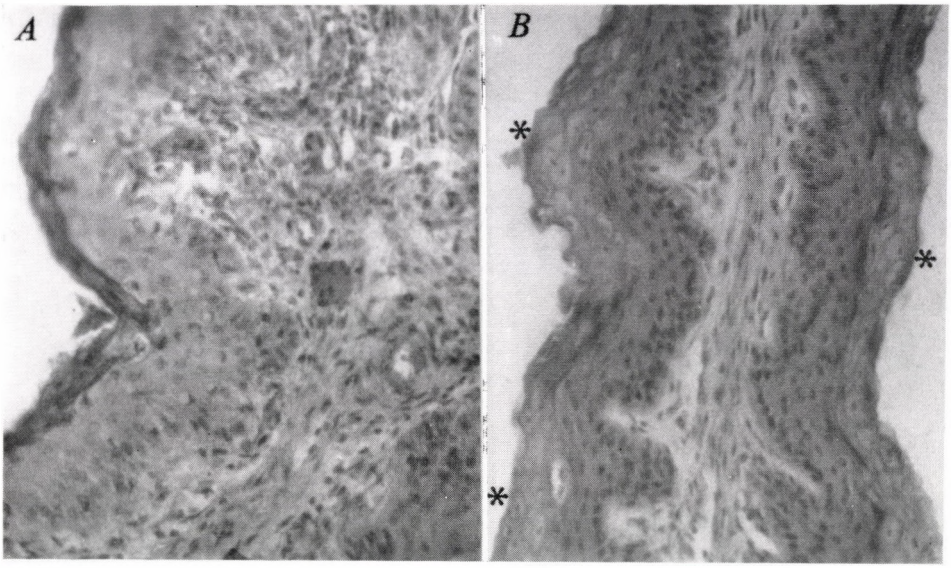

Fig. 5. Rumens from group 5 calves. (A) Compact structure of the stratified squamous epithelium and keratin layer. (B) Compact character of epithelium with clearly visible cells in the keratin layer (asterisks). H.-E., ×240

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COMPARISON OF DIFFERENT COMPUTATIONAL METHODS FOR MEASURING ANTIBODIES TO AVIAN INFECTIOUS BRONCHITIS VIRUS IN SINGLE SERUM DILUTION

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Twenty-eight one-day-old chickens with infectious bronchitis virus (IBV) maternal antibodies were immunized with strain H120 (Bronchovac-I, Phylaxia) in spray form. The chickens were kept in an isolator. On day 42 and 56 the chickens were immunized with IBV strain M41 ($10^{3\cdot0}EID_{50}$/0.1 ml). Serum antibody titres were measured by both serial dilution and single dilution ELISA on day 42, 56 and 76. "Twice negative average" (TNA), "sample to positive" (SP) and "subtraction method" (SM) titres were calculated from the serially diluted sera, and SP and SM titres were calculated from the single dilution. Titres obtained by the different methods showed a good correlation for sera of low, medium and high antibody levels. The authors recommend the use of the single dilution method.

Key words: Infectious bronchitis virus, IBV, enzyme-linked immunosorbent assay, ELISA, single serum dilution, titre calculation, immunization

The enzyme-linked immunosorbent assay (ELISA) was initially developed by Engvall et al. (1971) and van Weeman and Schuurs (1971). ELISA is a method widely used for detecting antibodies to avian infectious bronchitis virus (IBV) (Zellen and Thorsen, 1986; Thayer et al., 1987; Lana et al., 1983; Snyder et al., 1984; Garcia and Bankowski, 1980; Snyder and Marquardt, 1989; Monreal et al., 1985; Mockett and Darbyshire, 1981; Marquardt et al., 1981; Brown et al., 1990; Silim and Venne, 1989). Our aim was to develop an evaluation method for the single serum dilution infectious bronchitis ELISA that is easy to perform and gives results that are comparable between different laboratories.

Two major types of evaluation can be used for the infectious bronchitis ELISA (Zellen and Thorsen, 1986; Snyder et al., 1982; Lana et al., 1983; Snyder et al., 1984; Sacks et al., 1988; Garcia and Bankowski, 1980; Snyder and Marquardt, 1989; Monreal et al., 1985; Mockett and Darbyshire, 1981; Marquardt et al., 1981). A common characteristic of the two methods is that they give a titre value which is easy to interpret. The twofold serum dilution test is an easy but costly and labor intensive procedure. In this test, twofold dilutions of the test sera (from 1 : 20 to 1 : 2560), along with the similarly diluted positive and negative control sera are transferred to the test plate.

The average absorbance (OD) of the negative sera is calculated then doubled. This value is taken as the positivity limit. The last dilution of the test sera where the absorbance is higher than the calculated double average absorbance of the negative control sera is taken as the titre (twice negative average, TNA method).

A more efficient way of performing the ELISA is to work with single serum dilution (Snyder et al., 1982; Snyder et al., 1984).

Titre estimation using the "sample to positive" (SP) method

Titre estimation with calibration line is based on preliminary examinations. At least 40 sera with different titres must be examined by the twofold serial dilution method. Then a calibration line is calculated for the corrected absorbance readings and the titre values. To decrease variation between different plates, SP $\left(\text{sample to positive ratio, } \frac{Sample\ OD - Negative\ OD}{Positve\ OD - Negative\ OD}\right)$ ratio of a chosen dilution is calculated for each test serum. A dilution between 1 : 100 and 1 : 500 should be selected, since the near linear relationship that exists between the $\log_{10}$ of the corrected absorbance reading ($\log_{10}$SP) and the corresponding $\log_{10}$ of the observed titre (calculated by the TNA method) is more expressed within this dilution range (Snyder et al., 1983; Snyder and Marquardt, 1989).

Calculation of titre using the "subtraction method" (SM)

In the SP method, both the negative and the positive control sera are used for the titre calculation. The SM method differs from the SP method in two respects. Firstly, only the absorbance of the negative serum is used as internal standard. Secondly, the calibration curve is set up independently from the titres calculated by the TNA method, providing an independent way of obtaining titres.

Preliminary examination is necessary for determining the slope of the standard curve. Approximately 40 sera with different titres should be examined by the serial dilution method. Subsequently, for each serum the log(dilution) *(x)* values are plotted against the corrected log(absorbance) *(y)* values. To decrease variation betweens tests, the sample absorbance values are corrected by the substraction method: at each dilution, the absorbance of the negative sera is subtracted from the absorbance of the test sera (Snyder et al., 1982). A regression line is calculated for each test serum, then the common slope is determined. The resulting line reflects the near linear relationship between the log(dilution) *(x)* values and the corrected absorbance values of the test sera log(absorbance) *(y)* (Snyder et al., 1983; Snyder and Marquardt, 1989).

Materials and methods

Immunization

Twenty-eight one-day-old chickens with maternal antibodies were immunized with Atomist spray. The spray contained $10^{7.6}$ EID_{50}/m^3 lentogenic Newcastle disease virus NDV-6/10 of the VITAPEST® vaccine (Phylaxia) and $10^{4.5}$ EID_{50}/m^3 infectious bronchitis strain H120 of the Bronchovac-I vaccine (Phylaxia). The chickens were kept in isolator. Serum antibody levels were determined on day 42 (low-titre group).

On day 42, the chickens were immunized intranasally, intraconjunctivally and intratracheally with allantoic fluid containing IBV strain M41[1] (10^3 EID_{50}/0.1 ml). All sera were tested on day 56 (medium-titre group).

At the same time (day 56), the third immunization was carried out with strain M41 (oil-adjuvated vaccine, 0.5 ml s.c.). Antibody levels were checked on the day 76 (high-titre group).

Enzyme-linked immunosorbent assay (ELISA)

ELISA was performed as described, with some modification (Garcia and Bankowski, 1980; Engwall and Perlmann, 1971; van Weeman and Schuurs, 1971). As conjugate we used anti-chicken IgG (whole molecule, 1 : 15,000 dilution) produced in rabbits (Sigma Chemical Company, St. Louis, Missuri, U.S.A.). The substrate was TMB and the reaction was stopped with 4M H_2SO_4. The absorbancies were read at 450 nm on a Labsystems Multiskan® Plus reader then directly transmitted to an IBM computer.

Evaluation

In our single serum dilution system four wells each are used for the positive and negative control sera (A1–B2 and C1–D2, respectively). To increase accuracy, each test serum is measured into two wells, then the absorbancies are averaged. Having read the plate with a photometer, we used two different methods for converting the absorbance data into titre values. SP and SM titres were determined as described (Snyder et al., 1982; Mockett and Darbyshire, 1981).

Statistical analysis

The data obtained from the ELISA reader were transferred into a computer for analysis. Excel (Microsoft Corp., Redmond, Washington, U.S.A.) was used for arranging data, drawing preliminary curves and calculating re-

[1] Strain M41 is a gift from D. J. Alexander (Weybridge, U.K.).

Table 1

TNA titres and corrected absorbance data (SP method) at different dilutions for regression analysis

Sera	titre (TNA)	$\log_{10}$(titre) (TNA)	$\log_{10}$(SP) 1 : 80	$\log_{10}$(SP) 1 : 160	$\log_{10}$(SP) 1 : 320
1	160	2.204	−0.155	−0.229	−0.210
2	40	1.602	−0.511	−0.682	−0.481
3	80	1.903	−0.112	−0.250	−0.243
4	80	1.903	−0.139	−0.291	−0.278
5	160	2.204	−0.059	−0.089	−0.045
6	320	2.505	0.048	0.033	0.103
7	160	2.204	0.005	−0.114	−0.032
8	80	1.903	−0.219	−0.268	−0.230
9	160	2.204	−0.108	−0.130	−0.041
10	80	1.903	−0.264	−0.287	−0.157
11	320	2.505	0.001	−0.047	−0.056
12	320	2.505	−0.060	−0.116	−0.053
13	640	2.806	0.073	0.047	0.121
14	160	2.204	−0.101	−0.321	−0.374
15	160	2.204	−0.574	−0.356	−0.304
16	320	2.505	−0.036	−0.098	−0.012
17	160	2.204	−0.188	−0.243	−0.195
18	320	2.505	−0.009	−0.109	−0.156
19	1280	3.107	0.079	0.113	0.171
20	320	2.505	−0.019	−0.061	−0.063
21	160	2.204	−0.138	−0.116	−0.126
22	160	2.204	−0.169	−0.260	−0.260
23	160	2.204	−0.040	−0.111	−0.080
24	80	1.903	−0.244	−0.365	−0.418
25	160	2.204	−0.116	−0.195	−0.198
26	320	2.505	−0.019	−0.093	−0.042
27	320	2.505	0.047	0.002	−0.010
28	320	2.505	−0.064	−0.052	−0.053
29	160	2.204	−0.019	−0.057	−0.064
30	160	2.204	−0.148	−0.264	−0.242
31	320	2.505	−0.035	−0.036	−0.081
32	160	2.204	−0.078	−0.154	−0.186
33	80	1.903	−0.355	−0.427	−0.389
34	160	2.204	−0.141	−0.144	−0.125
35	320	2.505	−0.017	−0.065	−0.048
36	320	2.505	0.022	−0.006	−0.095
37	160	2.204	−0.233	−0.320	−0.383
38	80	1.903	−0.408	−0.403	−0.343
39	160	2.204	−0.134	−0.183	−0.182
40	320	2.505	−0.003	0.028	0.049
41	640	2.806	0.045	0.025	0.027
42	320	2.505	−0.137	−0.251	−0.220
43	160	2.204	−0.299	−0.442	−0.489
44	160	2.204	−0.178	−0.313	−0.391
45	80	1.903	−0.666	−0.643	−0.570
46	160	2.204	−0.246	−0.351	−0.386
47	320	2.505	−0.114	−0.121	−0.110
48	160	2.204	−0.246	−0.286	−0.340
49	160	2.204	−0.283	−0.294	−0.328
50	160	2.204	−0.324	−0.379	−0.454

gression lines. A program was developed in our laboratory, which directly receives absorbance data from the reader, averages, duplicates if necessary, then calculates titres by each method described in this paper.

Results

SP method

We determined the dilution where the corrected absorbance readings (SP method) best reflect the different titres determined by the serial dilution method. We selected dilutions 1 : 80, 1 : 160 and 1 : 320 and fitted a regression line to each set of data. Table 1 summarizes the data for the analysis of each selected dilution.

The results of the regression line calculation are shown in Table 2.

Table 2

Constants of the regression lines for the three selected dilutions

	Intercept	Slope	Correlation coefficient
1 : 80 dilution	−1.099	0.421	0.716
1 : 160 dilution	−1.323	0.495	0.811
1 : 320 dilution	−1.125	0.462	0.741

The highest correlation between $\log_{10}$ of the corrected absorbance reading ($\log_{10}$SP) and the observed titres (TNA method) was found at the 1 : 160 dilution. The calibration line for the 1 : 160 dilution is shown in Fig. 1.

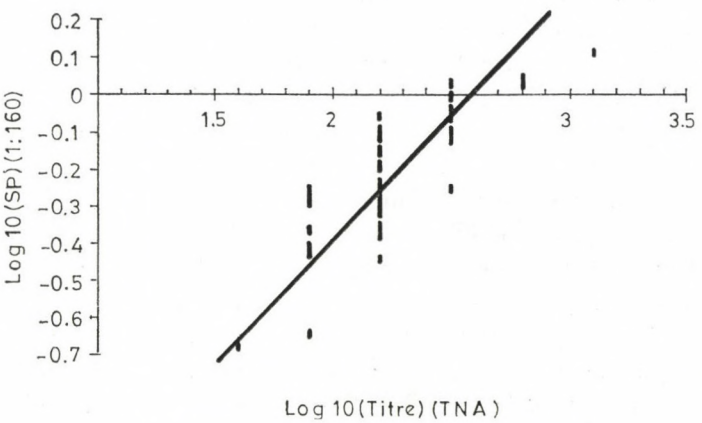

Fig. 1. Calibration line for the 1 : 160 dilution

The equation for the calculated 1 : 160 dilution regression line:

i. $y = b + a \times x$, that is

$$\log 10(SP) = intercept + slope \times \log_{10}(titre)$$

ii. $$\log 10(titre) = \frac{\log 10(SP) - intercept}{slope}$$

iii. $$\log 10(titre) = \frac{1}{slope} \times \log 10(SP) - \frac{intercept}{slope}$$

iv. Replacing the constants of the calculated 1 : 160 regression line:

$$\log 10(titre) = 2.020 \times \log 10(SP) + 2.672$$

v. $$titre = 10\ (2.020 \times \log 10(\mathrm{SP}) + 2.672)$$

Once the calibration line has been established, the equations (iv, v) can be entered into a microcomputer and sample titres can be calculated instantly. If any of the internal controls are changed, the calibration line must be re-calculated.

In our laboratory, a software was developed for IBM compatible computers that directly receives the absorbance data from the ELISA reader via a serial interface and immediately converts them to titre values. The program uses predetermined constants for calculating titres then stores the absorbance values that could be used for other statistical analyses or entered into a database later.

SM method

The positive–negative threshold (PNT) must be established. About 30 known negative sera must be repeatedly examined in serial dilution. The PNT is constructed by calculating the average absorbance value of the negative sera in each dilution then adding three standard-deviation-unit values. The values are further averaged to serve as the PNT. The resulting single PNT graphs as a straight line and represents the boundary reflecting the upper 0.5% of the negatives under an assumption of normality absorbancies (Snyder et al., 1982; Snyder et al., 1983). The PNT is shown in Fig. 2.

The titre of a test serum can be determined either graphically or algebraically. Graphically, $\log_{10}$ of the corrected absorbance (y value) of the serum is plotted against $\log_{10}$ of the working dilution (x value), then a line with the slope of the standard line is fitted through the point. The point where the fitted line intersects the PNT is considered the titre of the serum (Snyder et al., 1982; Sacks et al., 1988) (Fig. 3).

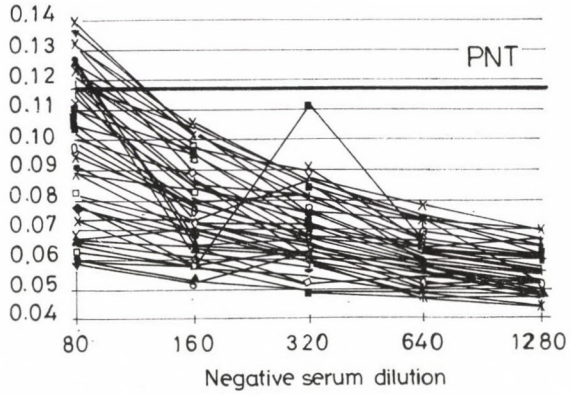

Fig. 2. PNT established with 18 sera in three repeated assays

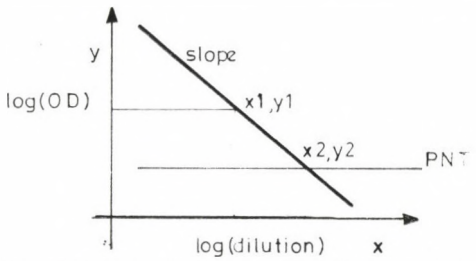

Fig. 3. Titre is determined graphically by fitting standard line (slope) to the assay point (x1, y1) and extending it through the PNT. The calculated x2 value of the intersection point is the log of the SM titre

If we solve the problem algebraically, the following equations can be set up:

i. Equation for the test serum:

$\log$ (corrected absorbance)$_{\text{test serum}} = \text{slope} \times \log$ (dilution) + intercept, that is

$y1 = slope \times x1 + intercept$

ii. Equation for the PNT line:

$log\ (PNT) = constants$, that is

$y2 = constants$

iii. The equation for the intersection point:

$log\ (PNT) = slope \times log\ (titre) + intercept$, that is

$y2 = slope \times x2 + intercept$

iv. From eq. no. *i* the intercept is:

$intercept = y1 - slope \times x1$, *then*

v. $y2 = slope \times x2 + y1 - slope \times x1$

vi. $y2 - y1 = slope \times x2 - slope \times x1$

vii. $y2 - y1 = slope \times (x2 - x1)$

viii. $\frac{y2 - y1}{slope} = x2 - x1$

ix. $x2 = \frac{y2 - y1}{slope} + x1$

x. $\log(titre) = \frac{\log(PNT) - \log(corrected\ absorbance)_{\text{test serum}}}{slope} + \log(dilution)$

Since the log (PNT) and the slope are constants, log(dilution) is the working dilution within the linear range of the standard line and log(corrected absorbance)$_{\text{test serum}}$ is the corrected absorbance reading, the titre of a serum can automatically be calculated with the help of a computer. The constants must be recalculated if any change occurs in the ELISA or if the internal negative control changes. If the appropriate constants are provided, the software described previously is also capable of calculating titres by the SM method.

Comparison of TNA, SP and SM titres

Sera from 28 chickens of different immune status were tested on day 42, 56 and 76 for antibody to infectious bronchitis by ELISA and the titres were calculated in five different ways. Each serum was tested by the serial dilution and by the single 1 : 160 dilution method. The three groups represented chickens with low, medium and high antibody levels (Fig. 4).

From the serial dilution, titres were calculated by the TNA, SP and SM methods. With the SP and SM methods, only the wells containing the 1 : 160 dilution (one well per serum) were used for the calculation.

When working only with 1 : 160 single dilution, titres were obtained by both the SM and the SP methods. In that case, two identical wells for the samples and four identical wells for both the control positive and negative sera were used for the evaluation.

Figure 4 shows the titres of chickens after the first (low titres), the second (medium titres) and the third (high titres) immunization. As can be seen, all five methods showed a distinct titre increase. Although the values obtained by the serial SM and the serial SP also correspond to the results of the other three methods, these calculations should not be used in the every-

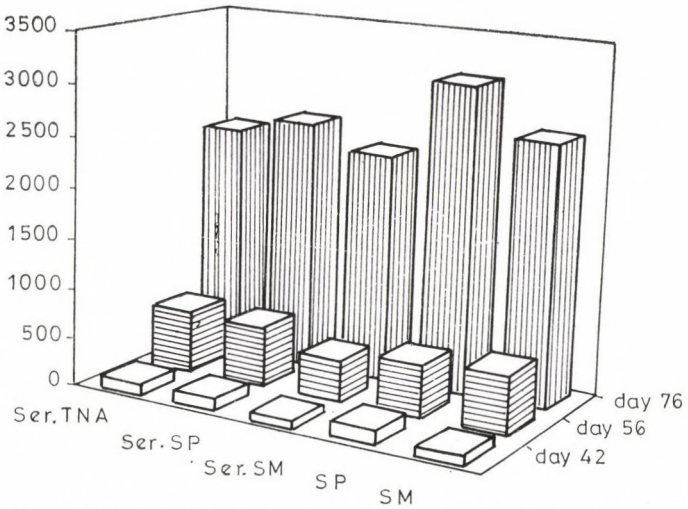

Fig. 4. Comparison of IBV ELISA titres of 28 chickens of different immune status

day practice since only one well per serum is used for the calculation, and inaccurate measuring of the reagents, plate defects, etc. can greatly influence the result obtained for the given serum.

Discussion

Different evaluation methods significantly differ in terms of efficacy and labour intensity. Using the twofold serial dilution method, 10 sera can be examined on one ELISA plate, assuming 1 : 20–1 : 2,560 dilutions. However, following natural infection or hyperimmunization the titres may exceed 1 : 2,560. In that case, only 6 sera can be checked for end titre on one plate.

In contrast to this, 44 sera can be titrated per plate when using the 1 : 160 single dilution SM or SP method, independently of the end-point titres. Both the SM and the SP methods are suitable for measuring low, medium and high antibody levels in the test sera (Fig. 4).

In addition to the trial described, sera from several other groups were analysed by the different methods. For the whole groups, titres calculated by the SM, SP and TNA methods consistently showed a linear relationship. Sometimes, however, marked titre differences were obtained for a given serum by the different methods, but this was attributed to plate defects or other technical problems. These errors could be eliminated by repeating the tests.

The question arises why we chose the 1 : 160 dilution (and not e.g. the 1 : 100 or 1 : 500). Firstly, we chose 1 : 160 because, of the three dilutions (1 : 80, 1 : 160 and 1 : 320) used, this was the dilution at which the highest

correlation ($r = 0.81$) occurred between the log(TMA) titres and the log(SP). Secondly, titres expressed from the 1 : 160 dilution are easily comparable with those of the serial dilution method.

The use of both the SM and the SP is recommended since they make the titre calculation more economical than the serial dilution method. With the twofold serial dilution method 10 sera can be tested on one ELISA plate, while running the 1 : 160 single dilution method 44 sera can be tested simultaneously. When using the SP method, one should be careful to determine the valid values for the positive control serum absorbancies since moderate changes in absorbance due to measuring errors, etc. can result in false titres. This applies, thought to a lesser extent, also to the negative sera.

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CHECKING OF IMMUNITY AGAINST NEWCASTLE DISEASE BY ELISA

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A total of 284 seven- to twelve-week-old Tetra SL chickens were assigned to 18 groups and immunized with a full or fractional dose of monovalent or multivalent inactivated vaccines subcutaneously or intramuscularly. Sera were taken from the birds at the time of challenge and tested for antibodies to Newcastle disease virus (NDV) by enzyme-linked immunosorbent assay (ELISA). The vaccinated birds were challenged with 10^6 ELD_{50} of virulent NDV subcutaneously. A close positive correlation ($r = 0.954$) was found between the protection percentage of the different groups and the group's arithmetical mean net extinction percentage (NE%) calculated from the net extinction values obtained for sera of birds belonging to the given group. NE % is easy to calculate and a good indicator of the flocks' immunity status. In our case: if NE% exceeded 80, the protection percentage of the flock was between 93.3 and 100%; if NE% was between 60 and 80, the protection percentage was between 75 and 87%, while with an NE% less than 60 the protection percentage ranged between 6.7 and 64.3%.

Key words: Newcastle disease, chicken, protection, ELISA

In the past decades, the degree of immunity to Newcastle disease (ND) was mostly determined by measuring the level of antibodies produced against the virus by the haemagglutination inhibiton (HI) test. Using the standardized test (Allan and Gough, 1974; Brugh et al., 1978), a close correlation was demonstrable between the HI titres measured at the time of challenge and the protection of birds against the challenge (Balla et al., 1976; Tóth and P. Juhász, 1978). For this reason, after the development of ELISA tests, most authors compared the HI titres of individual sera with the titres obtained by ELISA (Snyder et al., 1983; Marquardt et al., 1985; Thayer et al., 1987; Adair et al., 1989), or tried to establish correlations between the ELISA OD values and the HI titres (Miers et al., 1983). However, through the HI results only indirect correlations could be established between ELISA values and protection, and these correlations were only indirect indicators of the flock's immune status.

Only few papers have dealth with direct evaluation of the immune status of poultry flocks on the basis of ELISA values (Wilson et al., 1984). The evaluation used for characterizing the degree of protection has failed to find common use because of its complexity.

Commercially available kits can be used for measuring the immunity of birds and flocks only with the help computer programs sold together with the kits (Thayer et al., 1987).

This paper describes a simple evaluation method in which the group's arithmetical mean extinction values, calculated from ELISA extinction values of individual birds, enable a highly reliable assessment of the flock's protection against infection.

Materials and methods

Blood samples. Untreated serum samples collected at the time of challenge (21–28 days after immunization) from a total of 284 seven- to twelve-week-old Tetra SL chickens susceptible to ND, assigned to 18 groups and immunized subcutaneously or intramuscularly with a full or fractional dose of mono- or multivalent inactivated vaccines.

ELISA. The antigen was prepared as described previously (Goddard et al., 1988; Tekes et al., 1992). Polystyrol plates (E.I.A., Linbro, Flow Laboratories) were sensitized at +4 °C for 18 h with 0.05 ml of the antigen diluted 1 : 200 in a buffer (pH 5) prepared by mixing 0.1 M citric acid and 0.2 M Na_2HPO_4 at a ratio of 24 : 26. The plates thus sensitized were emptied and dried at room temperature, then stored at −20 °C. The plates prepared in that way were used for maximum 6 months.

Positive control serum. Untreated, pooled blood serum of 10 twenty-week-old SPF chickens immunized with a full dose of a monovalent, inactivated vaccine against ND was used as positive control serum (HI titre: 1 : 128).

Negative control serum. Untreated, pooled blood serum from 10 forty-week-old SPF hens was used as negative control serum (HI negative).

Washing solution and diluent. 0.01 M phosphate buffer (pH 7.2) containing 0.5 M NaCl and 0.05% Tween 20. When diluting the conjugate, this solution was supplemented with 10% horse serum.

Conjugate. Anti-chicken IgG (Sigma) labelled with peroxidase enzyme.

Substrate. Four mM tetramethylene benzidine (TMB, Serva) and 0.02% H_2O_2 was dissolved in 0.1 M acetate buffer (pH 5.5).

Arresting solution. 4N H_2SO_4.

ELISA procedure. Before use, the sensitized plates were stored for 1 h and then washed three times by filling up and emptying the wells. We measured 0.05 ml of the 1 : 100 diluted test sera into two wells each and 0.05 ml aliquots of the 1 : 100 diluted negative and positive sera into four wells each of the plate. Subsequently, the plate was incubated at +37 °C for 30 min. The plate was washed three times, 0.05 ml of the appropriate dilution of the conjugate was added to the wells, and then the plate was incubated at +37 °C

for 1 h. After three cycles of washing, 0.05 ml of the substrate solution was measured into the wells. The reaction was stopped after the desider colour intensity had developed (approx. 5 min). The colour reaction was measured at 450 nm with a Titertek Multiskan R Plus photometer (Labsystems).

Challenge infection. On postvaccination day 21–28, the 284 birds assigned to 18 groups were challenged subcutaneously with 10^6 ELD_{50} of virulent NDV. The chickens were kept under observation for 14 days after the challenge. Birds that died were subjected to postmortem examination to determine whether death had been caused by ND.

Evaluation of data. The mean extinction (ME) value of the flocks was obtained by calculating the arithmetical mean of the extinction values (E) measured at 450 nm for sera of binds belonging to the different flocks. Net extinction percentage (NE%) was calculated by the following for mula:

$$NE\% = \frac{\text{Flock ME} - \text{negative standard E}}{\text{Positive standard E} - \text{negative standard E}} \times 100$$

where negative and positive standard E = the arithmetical mean of extinction values obtained for the negative and positive serum measured into four wells each.

Protection was expressed in per cent, by relating the number of birds living and symptomless on postchallenge day 14 to the total number of birds challenged.

Results

The arithmetical means of net extinction values obtained for the different flocks, the standard deviation observed within the flocks, and the protection percentage of the flocks after challenge are showing in Table 1. The regression line calculated from the summarized data by linear regression is presented in Fig. 1 (the equation of the line obtained by us was $y = 0.861x - 2.207$). The coefficient of correlation obtained for the above data by linear correlation performed for the 18 flocks is 0.954.

Discussion

The failure of ELISA procedures to gain widespread use in the case of numerous diseases was primarily accounted for by the fact that the results obtained by methods developed by different authors could not be compared. This was due to the lack of standardization of positive and negative sera used in the test and to the use of a variety of different evaluation methods.

Table 1

Net extinction values and postchallenge protection of chickens by flock

Flock no.	Number of chickens	Net extinction ±SD%	Protection, %
1	14	25±15.68	35.7
2	15	74±19.57	86.7
3	15	30±11.35	60
4	30	22±12.33	40
5	10	94±9.07	100
6	10	70±17.79	80
7	15	34±6.78	20
8	15	37±8.85	46.7
9	15	57±10.48	64.3
10	20	64±18.1	75
11	15	10±2.1	6.7
12	15	20±5.45	26.7
13	15	88±9.31	100
14	15	81±10.22	93.3
15	15	16±7.54	33.3
16	15	5±1.73	6.7
17	20	71±13.29	85
18	15	63±14.75	86.7

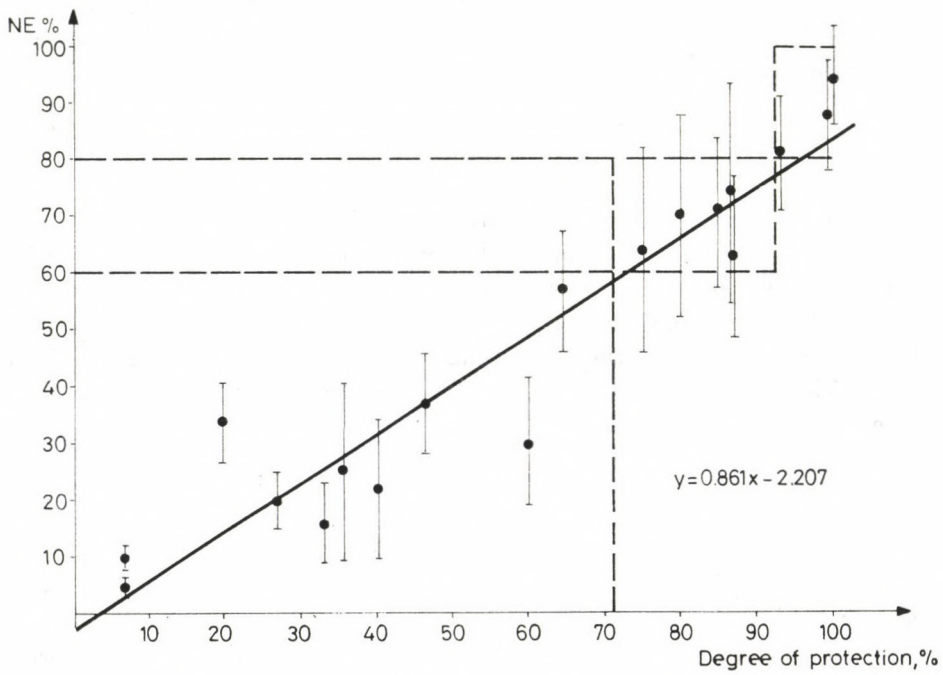

Fig. 1. Regression line for the correlation of net extinction values (NE%) measured in the different flocks with the degree of protection

Studies using different kits have revealed that ELISA values better reflect the immune status of animals than do results of the HI test (Marquardt et al., 1985; Adair et al., 1989). This has increasingly called for methods reliably expressing the correlation of ELISA values and protection against infection without complex calculations.

Snyder et al. (1983) found a close positive correlation ($r = 0.0965$) between HI titres and titres obtained by ELISA. The same correlation was $r = 0.94$ and $r = 0.98$ according to Marquardt et al. (1985) and Thayer et al. (1987), respectively. A close positive correlation was demonstrated between the net extinction value and the ELISA titre as well (Snyder et al., 1983). On the basis of that correlation, the ELISA titres are calculated, with the help of a formula, from the extinction value obtained for a single serum dilution.

Wilson et al. (1984) characterized the extinction values by whole numbers extending from 0 to 9, calculated on the basis of extinction values obtained for the positive serum. A close correlation was established between the values thus obtained and the survival rate of chickens.

As the HI titres are a good indicator of the birds' immune status, the ELISA titres are in close correlation with the HI titres and the extinction values with the ELISA titres, we thought that the extinction values must also show a satisfactory correlation with the degree of protection.

On the basis of the results reported by Snyder et al. (1983) and Wilson et al. (1984), in this study we tried to find a direct correlation between the extinction values and the degree of protection which could be expressed in a simple way and would be typical of the group tested.

We expressed the arithmetical mean of the group's net extinction value calculated from extinctions of the individual birds' serum samples as per cent of the net extinction measured for the positive serum used by us (NE%). A close positive correlation ($r = 0.954$) was established between the NE% and the degree of protection obtained for the given group. Using the evaluation adopted by us (NE%), by ELISA tests of groups comprising 15–30 birds we obtained sufficient information about the immunity of the different groups. On the basis of the data shown in Fig. 1, the groups tested were assigned to one of three categories. If NE% exceeded 80, the degree of protection of the flock was between 93.3 and 100%; if NE% was between 60 and 80, the protection percentage was between 75 and 87%, while with an NE% less than 60 the protection percentage ranged between 6.7 and 64.3%.

In our opinion, the groups belonging to the first category are well protected against infection, those assigned to the second category have satisfactory protection, while the groups belonging to the third category have insufficient protection.

The protection of groups assigned to the second category must be checked by regular tests performed every three weeks, or the groups must be revaccinated. When used in conjunction with the evaluation method described above, the ELISA test is considered suitable for checking the immunity of flocks and possibly for replacing the labour-intensive HI test used at present.

The availability of international standard negative and positive sera would also allow the comparison of tests performed by different laboratories.

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PATHOGENIC PROPERTIES OF NEWCASTLE DISEASE VIRUS ISOLATES IN THE SUDAN

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Six Newcastle disease virus (NDV) isolates were obtained from disease outbreaks on different poultry farms in the Sudan between 1988 and 1991. The pathogenic properties of these isolates were studied in comparison to those of strain Herts 33/56. All the isolates were similar in that they killed chicken embryos quickly, in mean death time (MDT) and embryo lethal dose 50 per cent (ELD_{50}), had higher intracerebral pathogenicity indices (ICPI), and produced viscerotropic lesions in the infected chickens. The field isolates had the characteristics of the velogenic viscerotopic strains of NDV. The pathogenesis of infection caused by one of the isolates was studied. The virus was first detected in different organs and in oral and cloacal swabs on the third day after infection.

Key words: Newcastle disease virus, pathogenicity, properties, Sudan

The presence of Newcastle disease (ND) in the Sudan was first reported in 1951 (Anon., 1951). Since then the disease has been regularly mentioned in all reports of the Sudan Veterinary Services. Diagnosis was based on the clinical signs and postmortem findings as well as the general picture of the disease, but the virus was isolated and identified for the first time by Karrar and Mustafa (1964). Yet, apart from the work done by Ballouh et al. (1983), the characterization of the local isolates has not been published so far. This work is aimed at throwing light on the pathogenic properties of some NDV isolates in the Sudan on the basis of works carried out between 1988 and 1991. The pathogenic properties of one local NDV isolate in chicken were also studied.

Materials and methods

Chickens and embryonated hen's eggs. Chickens and embryonated hen's eggs were obtained from a Hisex flock raised on the poultry farm of the Central Veterinary Research Laboratory (CVRL). The flock is known to be free from NDV, salmonellas and mycoplasmas. Serum samples were collected from experimental chickens and tested for NDV antibodies before use.

Virus strains. Six virus strains were isolated in embryonated hen's eggs from brain, spleen and lung samples collected from field outbreaks of suspected ND. The dates and places of the outbreaks, together with the history

Table 1

Origin of NDV field isolates, date and place of the outbreaks, age and vaccination history of affected chickens

Isolate	Date of the outbreak	Place of the outbreak	Age of the affected birds (weeks)	Vaccination against ND
TY-1/90	July 1990	Tayba, 30 km south of Khartoum	6	+
Soba-89	July 1989	Soba, 20 km southeast of Khartoum	3	—
Atb-88	June 1988	Atbara, Northern State, 300 km north of Khartoum	40	—
Dik-90	Sept. 1990	Dikheinat, 15 km south of Khartoum	56	+
TY-2/91	Feb. 1991	Tayba, 30 km south of Khartoum	7	+
Bag-90	Oct. 1990	El Bagair, 35 km south of Khartoum	5	+

of the affected flocks, are shown in Table 1. The isolates were identified by the haemagglutination inhibition (HI) test against a known antiserum (Weybridge, England), using a standard technique (Allan et al., 1978). Each isolate was passaged in embryonated eggs twice. Allantoic fluid was collected from the dead embryos and stored at −70 °C for subsequent experiment. NDV strain Herts 33/56 was used for comparison.

Determination of embryo lethal dose 50 per cent (ELD_{50}) *and mean death time* (MDT). The ELD 50 per cent endpoint and MDT were determined as described by Allan et al. (1978).

Determination of intracerebral pathogenicity index (ICPI). A total of 10 day-old chicks were inoculated intracerebrally with 0.05 ml of fresh, 1 : 10 diluted allantoic fluid of each isolate and of the Herts strain. Two control chicks received sterile normal saline. The birds were observed daily for 8 days and the ICPI was calculated as described by Allan et al. (1978).

Determination of pathogenicity to 8-week-old chickens. A total of 42 eight-week-old chickens were used. Each field isolate and the Herts strain were separately used for infecting 4 chickens by swabbing undiluted fresh allantoic fluid onto the conjunctiva and cloaca of each bird. Two birds were housed separately as control in each experiment. Clinical signs were observed daily for 10 days unless the chick died. The time of death and the postmortem findings were recorded. Differentiation of the isolates was done by the procedure of Hanson (1980).

Pathogenesis of infection caused by a field isolate in chicken. The field isolate designated TY1/90 was selected for infecting 6-week-old chicks, and virus shedding was monitored by oral and cloacal infection. A group of 27 birds were inoculated intranasally with a dose of $10^{7.5}$ ELD_{50} per bird. Three birds were sacrificed every 24 hours and the brain, spleen, lungs, trachea and intestine of the birds, as well as oral and cloacal swabs were collected aseptically for virus reisolation attempts.

Results

Six isolates of NDV were obtained from field outbreaks that occurred between June 1988 and February 1991 (Table 1). All the isolates agglutinated chicken erythrocytes and their haemagglutinating ability was inhibited by a known NDV-positive antiserum. The isolates grew readily in embryonated hen's eggs to titres ranging between $10^{8.1}$ and $10^{10.2}$ ELD_{50}. The inoculated embryos died with haemorrhages in various organs.

Table 2 shows some of the properties of the isolates, including ELD_{50}, MDT and ICPI.

Table 3 presents the clinical and postmortem findings that occurred after inoculating 8-week-old chickens with the six NDV isolates and NDV strain Herts. The incubation period was 3–5 days and the onset was sudden. Diarrhoea eye oedema and paralysis were marked. Death was abrupt and occurred 4–6 days after infection.

Table 2

Some pathogenicity indices of NDV field isolates and strain Herts 33/56

Virus isolate	ELD 50%[1]	MDT/MLD[2]	ICPI[3]
TY-1/90	$10^{8.5}$	49.6	1.92
Soba-89	$10^{9.3}$	52.8	1.81
Atb-88	$10^{10.1}$	48.6	1.73
Dik-90	$10^{9.1}$	49.0	1.65
TY-2/91	$10^{8.1}$	50.0	1.78
Bag-90	$10^{9.1}$	45.7	1.72
Herts 33/56	$10^{10.2}$	48.0	1.80

[1]Embryo lethal dose 50% endpoint; [2]Mean death time (MDT) for the minimum lethal dose (MLD); [3]Intracerebral pathogenicity index

Table 3

Clinical and postmortem findings after infection of 8-week-old chickens with NDV field isolates and strain Herts 33/56

Virus	Mean incubation period (days)	MDT (days)	Eye oedema	Paralysis	Diarrhoea	Sick	Dead	Gut haemorrhages	
								Proventriculus	Intestine
TY-1/90	4	5	2/4*	1/4	4/4	4/4	4/4	3/4	3/4
Soba-89	4	4.7	4/4	0/4	3/4	4/4	4/4	4/4	4/4
Atb-88	4	5	3/4	1/4	4/4	4/4	4/4	0/4	4/4
Dik-90	3.7	5	3/4	1/4	4/4	4/4	4/4	3/4	4/4
TY-2/91	4	5	0/4	3/4	2/4	4/4	4/4	0/4	2/4
Bag-90	4	5.5	1/4	0/4	4/4	4/4	4/4	2/4	1/4
Herts 33/56	2	3.7	0/4	0/4	3/4	4/4	4/4	4/4	4/4

*Number of birds showing signs or lesions/total number of birds; MDT = mean death time

Table 4

Detection of virus in organs and swabs of chickens inoculated with NDV isolate TY-1/90

Days post inoculation	Brain	Spleen	Lung-trachea suspension	Intestine	Oral swabs	Cloacal swabs
1	—	—	—	—	—	—
2	—	—	—	—	—	—
3	—	+	+	+	—	—
4	+	+	+	+	+	+
5	+	+	+	+	+	+
6	+	+	+	+	+	+

The results of NDV reisolation attempts from organs and swabs of chickens that were inoculated with isolate TY1/90 are shown in Table 4. The virus was first detected on postinoculation (PI) day 3 in the spleen, lung-trachea suspension and intestine. From PI day 6, when all the remaining chickens died, the virus persisted in all the organs tested and in the oral and cloacal secretions.

Discussion

The six isolates studied in the present work were originally obtained from natural outbreaks of ND involving vaccinated and unvaccinated chicken flocks of different age. The affected birds showed signs suggestive of digestive and nervous system involvement. The natural disease picture and the pathogenicity of the six isolates were comparable. All six isolates were similar in that they killed chicken embryos quickly and produced viscerotropic lesions in infected chickens. These findings, together with the MDT and ICPI values of the six NDV isolates as compared to those of Herts strain 33/56, indicated that the local strains were velogenic pathotypes. The isolates seemed to be closely related to each other and were comparable to NDV strain Herts 33/56.

Recovery of the field isolate of NCV from more than one organ of experimentally inoculated chickens indicated the pantropic nature of the virus. Brain, spleen, lung, trachea and intestine as well as oral and cloacal swabs are all suitable for virus isolation during the acute stage of the disease.

Both the disease picture seen in natural outbreaks and in experimentally infected chickens and the pathogenic properties of the isolated NDV strains were the same as those described by Hanson et al. (1973) for the viscerotropic pathotype of NDV. The haemorrhagic visceral lesion produced by NDV was previously reported from the Sudan (Khogali, 1971; Eisa, 1979; Gaffar Elamin et al., 1981). Ballouh et al. (1983) studied some properties of NDV strains

isolated between 1963 and 1979. Eight out of 12 isolates were found to be velogenic and 4 were mesogenic.

In conclusions, it can be established that velogenic viscerotropic NDV is the most prevalent NDV strain in the Sudan and the strain responsible for the heavy annual losses of the country's poultry industry.

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BOOK REVIEWS

J. ECKERT, E. KUTZER, M. ROMMEL, H.-J. BÜRGER, W. KÖRTING; *Veterinärmedizinische Parasitologie, begründet von Boch, J. und Supperer, R.* (Veterinary Parasitology, founded by Boch, J. and Supperer, R.). 4th edition, Verlag Paul Pary, Berlin and Hamburg, 1992. 920 pages, with 254 illustrations and 68 tables. Price: DM 158.—

The first edition of Boch and Supperer's "*Veterinärmedizinische Parasitologie*" was published in 1971 and was regarded a most up-to-date textbook dealing with parasitic infections of the main domestic animal species in separate chapters and providing the user with many relevant sources of references at the end of sub-chapters. That excellent book of 408 pages included 106 illustrations, most of which were original photographs, and 12 tables. The second edition in 1977 amounted to 517 pages, 160 illustrations and 20 tables. It was in 1983 that the 3rd edition of Boch and Supperer's Veterinary Parasitology was published, then with the cooperation of Professor J. Eckert (Zürich), E. Kutzer (Vienna) and M. Rommel (Hannover). That book ran to 533 pages, included 192 illustrations and 28 tables, and contained more information on parasitic diseases of wild animals, rabbits and bees.

The former associates, together with Professor H. J. Bürger (Giessen) and W. Körting (Hannover), have now entered on Boch's and Supperer's inheritance, intending to prepare a comprehensive textbook of veterinary parasitology both for students, teachers and veterinary practitioners in German-speaking countries. The scope by which they extended the book (920 pages, 254 illustrations, 68 tables) itself illustrates the enormously increased amount of information included in the recent edition.

Having summarized the most important definitions and abbreviations on 4 pages, a general, 100-page long chapter follows dealing in sub-chapters with the systematics and taxonomy of protozoa and metazoa; host-parasite relationship (susceptibility, resistance, immunity, etc.); pathogenesis of parasitic infections (factors of pathogenicity and virulence in parasites, biological and ecological background, mechanic-toxic-lytic-immunobiological effects); diagnostic procedures; peculiarities of the epidemiology of parasitic infections; epidemiology and the basis of parasite control; a most comprehensive review of the effect of parasites on the environment. There is a recent, exhaustive bibliography attached to each sub-chapter. Included in this chapter, 16 tables contain many data usefully amplifying the text. This concise chapter replaces to some extent and updates topics which used to be discussed in a separate book (Th. Hiepe, R. Buchwalder and R. Ribbeck: "Allgemeine Parasitologie" (General Parasitology), Fischer Verlag, Stuttgart, New York, 1981).

The contributors, faithfull followers of the founders' book structure, continued to deal with parasitic infections in the order of host species. To chapters on ruminants (255 pages), horses (79 pages), pigs (73 pages), dogs and cats (128 pages), rabbits (18 pages), poultry (85 pages), wildlife (ruminants, wild hog, hare, pheasant and partridge; 22 pages), new chapters on hedgehogs (6 pages) and fish (63 pages) are added. In addition to the traditionally specified interactions caused by protozoa, helminths and arthropods in different host species, recently emerged infections (e.g. neosporosis in carnivores and ruminants, cryptosporidiosis in various species, sphaerosporosis in fish, etc.) are also discussed. The enormous amount of literature had inevitably to be selected. Thus, many references from the earlier editions were omitted to give room for recent ones; the authors of various chapters made the selection in a highly professional way. Anyone interested in the incidence of a specific parasite infection, in epidemiology, morphology and life cycle, pathogenesis, diagnosis, therapy, control or public health significance will find the book a wealth of relevant references. The general index and 2 appendices, 36 pages long, include in alphabetical order both trade names and generic names of the antiparasitic

drugs used in different host species, referring also to their registration stage in German-speaking countries as well as the name of the manufacturers — a useful means of quick orientation.

Only a few statements can be traced in the textbook which might perhaps be argued (e.g. the validity of *Eimeria mivati; E. tenella:* maximum of oocyst output approx. 10 days after infection).

The acceptance of the nomenclature guidelines (SNOAPAD) both of the World Association for the Advancement of Veterinary Parasitology and the World Federation of Parasitologists and the consistent use of "-osis" to denote parasitic infections will certainly contribute to bringing the long dispute on this issue to an end. The text is clearly written, well illustrated and edited with care. The tremendous amount of information brought together on parasites and parasitic infections by the successor team of authors makes the book a worthy, updated continuation of the previous editions. It highly deserves room on the bookshelves of not only veterinary students, but also veterinarians, parasitologists, pathologists, immunologists, pharmacologists, specific legislative corporations, conservationists and all biologists who work with any topic of, or are interested in, the particular way of life related to parasitism.

István VARGA

Clyde A. KIRKBRIDE: *Laboratory Diagnosis of Livestock Abortion.* Third edition. Iowa State University Press, Ames, Iowa, 1990. 274 pages, illustrated, paperback. ISBN 0-8138-1593-2. Price: USD 26.95.

The third edition of "*Laboratory Diagnosis of Livestock Abortion*", the work of Dr. C. A. Kirkbride and his 35 co-authors, is a perfect manual both in its contents and presentation. In addition to emphasizing the most important diagnostic aspects of abortion by animal species, it provides the reader with a special knowledge comprising all the details of laboratory work.

The book is an indispensable reference primarily for specialists of pathological anatomy, histopathology and their complementary laboratory examinations. Its clear arrangement and the theoretical and practical knowledge provided on abortion in different animal species make the book an attractive and valuable reference for veterinary students as well. These features make the book suitable for arousing the interest of readers less familiar with the narrower special field and, thus, for winning over talented students for routine diagnostic and research activity at veterinary institutes.

Laboratory Diagnosis of Livestock Abortion is similarly useful for veterinary practitioners. By describing the deficient symptomatology preceding abortions and the characteristic but nonspecific lesions occasionally shown by the fetuses and fetal membranes, the authors emphasize the necessity of laboratory examinations. At the same time, the readers receive accurate information on the correct evaluation of diagnostic findings.

Already the introductory chapters are indicative of a specialist who has dealt with the diagnosis of abortion for a long time and on a high level. The importance of the correct time and method of sample collection and shipment to the laboratory cannot be overemphasized. The objective evaluation of gross pathological changes and the expert performance of histopathological examinations are none the less important.

The authors give a detailed description of the numerous factors that may be involved in the aetiology of infectious and non-infectious abortions. By listing these agents and describing their effect, the authors indicate that some of the abortifacients are either no longer detectable at the time of abortion or they exert their effect indirectly; thus, their presence can be indicated by cytological or other fine structural examinations or by the high levels of antibodies produced against them. By writing down all these facts, Professor Kirkbride furnishes an explanation for the low (30–40%) success rate of diagnostic evaluation of aborted fetuses and fetal membranes submitted for laboratory examination all over the world.

It is very useful that Professor Kirkbride has devoted a separate chapter to the evaluation of serological examinations during abortion, as that evaluation is often inconsistent because of the introduction of new methods, the use of locally prepared reagents, and the varying immunological knowledge of the specialists.

Its constant effort to aid the identification of primary aetiological agents involved in causing abortion is a great advantage of the book. It is very useful that the specific tests suitable for determining the pathogenicity of different bacterial strains and the procedures for detecting viruses implicated in abortion are described separately by disease and by patho-

gen. Detailed data are given on the preparation of reagents and solutions, making the diagnostic tests sufficiently reproducible.

In the general overview, the author points out the possible incidence of concomitant infections, or includes works in the reference list that inform the reader about such infections. The highly professional and rational selection of references deserves special mention.

It is a pity that the description of efficient and specific microbiological examinations of semen samples is missing from the chapter devoted to laboratory examinations (of fetal organs, stomach contents and placenta, as well as the blood and vaginal secretion of the aborted female). Namely, despite the stringent semen control measures adopted with the spread of artificial insemination, experience shows that semen still plays a role in transmitting infection.

The part dealing with complementary examinations unfortunately lacks a description of methods serving for the evaluation of milk samples. Such tests may be necessary in connection with sporadic or mass abortion occurring in certain cattle herds or sheep flocks, when excretion of pathogens via the milk must be determined in a certain part of the stock or on herd level.

In summary, it can be stated that the book edited by Professor Kirkbride is an indispensable tool for laboratory specialists all over the world. It provides both beginners and experienced specialists with theoretical and practical guidelines for detecting, and evaluating the role of, pathogenic agents that play a role in abortion and reproductive diseases of livestock.

Marietta RÁDY

Klaus BICKHARDT: *Kompendium der allgemeinen inneren Medizin und Pathophysiologie für Tierärzte.* First edition. Verlag Paul Parey, Berlin and Hamburg, 1992. 231 pages, 15 tables and 55 figures, paperback. Price: DM 36.—

The author recommends the book first of all to veterinary students and veterinary practitioners. The textbook character is indicated by the fact that the work was published in the Parey Studientexte series. According to the author's introduction, the book is based on many years of experience and the written sketches of his lectures, and its principal aim is to serve as an aid to students in following the lectures. Accordingly, the book does not include the whole scope of veterinary pathophysiology; at the same time, it contains many species-specific pathophysiological characteristics typical of domestic animal species (horses, cattle, swine, carnivores). The work is divided into 19 chapters and, within the latter, numerous subchapters. It has a clear and concise structure. Decimal classification within the chapters enables rapid reader orientation.

The first five chapters deal with the regulation and pathological processes of the organism's internal milieu, cellular metabolism, and the abnormalities of the blood elements. The subsequent chapters follow a classification by organ system (respiration and respiratory metabolism, heart and blood circulation, musculature, liver, digestive organs, urinary tract, central nervous system), or are devoted to specific topics of pathological metabolism and regulation (general adaptation syndrome, stress, protein metabolism and enzymopathies, carbohydrate and lipid metabolism, fluid balance, acid–base balance, bony system and mineral metabolism, thermoregulation).

The detailed *tables* at the end of the book contain the most important clinical reference values of domestic animals, the reference values of certain laboratory variables of the blood, and the factors of conversion from conventional to SI values. The questions given at the end of the book, relating to the different chapters, enable students to check their own knowledge.

The different pathophysiological mechanisms and the mechanism of pathological regulation are illustrated by didactic *illustrations* which help the readers understand the text. Some of the figures appear rather complicated on first inspection, while a few others provide an incomplete illustration of pathological processes. The book contains no photographs, obviously to reduce the price, as the work is intended for students. For the reviewer, a special feature of the book is that it provides, besides the pathophysiology of domestic animals, a brief symptomatology of the different diseases; moreover, the possible methods of treatment are also described concisely at the end of the individual chapters.

A great advantage of the book for veterinary students is that it provides guidelines about the pathological functioning of organs and organ systems of several animal species. It can be a useful reference for veterinary practitioners who wish to brush up their knowledge

of pathophysiology. As the work is a textbook rather than a manual, it is quite understandable that readers wishing to go more deeply into the different topics must consult other manuals.

In summary, it can be stated that the book is a useful reference for veterinary students, as well as for students of agricultural and biological sciences, in learning the subject-matter of pathophysiology. In addition, it provides a brief insight into veterinary internal medicine, giving a concise description of the most important clinical signs resulting from the pathomechanism of different diseases. Veterinary practitioners get an overview of the pathogenesis of diseases of the most important domestic animal species and of the essence of pathological processes underlying the disease symptoms.

Károly VÖRÖS

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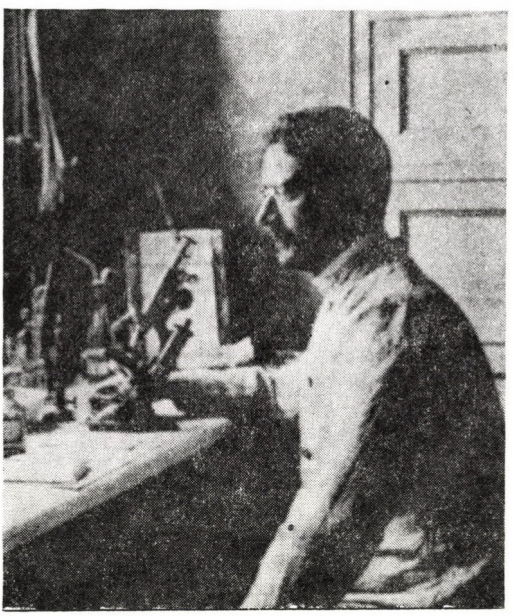

Dr. Aladár AUJESZKY (1869–1933),
in 1902, the year of the discovery of the disease

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