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FEEDING TRIAL IN PIGS WITH A DIET CONTAINING SODIUM *n*-BUTYRATE

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Pigs weighing 7 to 102 kg were fed a diet containing 0.17% sodium *n*-butyrate. The diet increased the average daily body mass gain of pigs by 23.5%. Due to its dietetic effect, feed consumption increased by 8.9%. However, owing to the higher feed conversion, specific feed utilization was reduced by 11.8%. The experimental diet markedly reduced the percentile proportion of coliform bacteria in the ileum as compared to *Lactobacillus* ssp.: it decreased the coliform count and increased the counts of *Lactobacillus* spp. The diet increased the length of ileal microvilli and the depth of caecal crypts. It raised the concentration of immunoreactive insulin in the blood plasma. The feed supplemented with sodium butyrate did not alter adversely the clinical indices tested. It reduced feed costs by 9% and increased the returns from sales by 13%. As the additive is normally produced by microbial fermentation in the large intestine, it is not alien to the body. Sodium butyrate exerted its favourable effect in 3.6- to 24.2-fold lower concentrations than the organic acids (citric acid, fumaric acid, propionic acid) used earlier.

With respect to its favourable biological and economic effect, sodium *n*-butyrate can be recommended for use in pig feeding as a growth promoter.

Keywords: Butyrate, pig, body mass gain, intestinal microflora, clinical indices

It has been observed that piglets fed a diet preserved or supplemented with organic acids show higher body mass gain and improved feed utilization (Cole et al., 1968; Cornelius, 1988; Edmonds et al., 1985; Falkowski and Aherne, 1984; Giesting and Easter, 1985; Kirchgessner and Roth, 1982; Kutas et al., 1973). Although the biological role of butyric acid is in many respects the same as that of the organic acids used earlier (citric acid, fumaric acid, propionic acid and lactic acid), its dietetic effect in pigs has not been studied yet. The aim of the present experiments was, therefore, to study the effects exerted by a butyrate containing feed on the clinical status, feed utilization, and body mass gain of pigs. As butyric acid is known to regulate cell division, we monitored its effect on the composition of the intestinal microflora, the development of oesophagogastric ulcer, and the structure and behaviour of the intestinal epithelium in pigs.

Materials and methods

Experimental animals and diet. The experiments were carried out in a large pig unit. A total of 164 Large White Landrace × Duroc piglets (average body mass: 7 kg) weaned at 31 days of age were used. The piglets were kept in a two-storey battery with a capacity for 164 piglets, in groups of six. The battery was designed and manufactured by the Joint Venture for Industrial Pork Production (ISV). Piglets kept in the same air-space were divided into two groups of identical size and total liveweight (control group: 82 piglets, total liveweight: 574 kg; experimental group: 82 piglets, total liveweight: 574 kg). The piglets remained in the battery for a total of 79 days. After the second weighing they were transferred to the fattening house, where both the control and the experimental groups were subdivided into two groups of about 40 piglets each. The piglets were fattened for 139 days until they exceeded the liveweight of 100 kg. The animals were kept according to the ISV management technology that had been used on the farm already earlier, and were fed diets and concentrates formulated according to the specifications of Agrokomplex–Central Soya (Agrokomplex, Agárd). In the battery the piglets received a piglet diet for a month, then a piglet starter diet. After

Table I

Composition (in %) of diets fed during the trial

Ingredients	Piglet diet (TA 8030)	Piglet diet (TA 8099)	Piglet rearing diet (TA 8012)*
Sodium *n*-butyrate	0.17	0.17	0.17
Maize	62.83	76.83	84.33
Soybean (48%)	10	15.5	8.0
Fish meal (70%)	5.5	2	—
Wheat flakes	9.5	—	—
Wheat bran	—	2	—
Sunflower meal (1st class)	—	—	4
Piglet diet supplement	12.0*	—	—
Complete premix	—	3.5**	3.5***
	100	100	100
Contents			
Metabolizable energy (MJ/kg)	13.53	12.91	12.50
Crude protein (%)	21.10	18.02	16.05
Crude fat (%)	5.10	2.99	2.98
Crude fibre (%)	2.14	2.58	3.90

* KE 8030 000 piglet diet supplement; ** KP 8099 000 piglet starter complete premix; *** KP 8120 000 pig grower complete premix. The supplements and complete premixes marked with asterisks are the products of Central Soya (Agrokomplex, Agárd). + containing 15.75 mg/kg Tylan.

transferred to the fattening house, they continued to receive the piglet starter diet for a week, then they were fed a grower diet produced at 10-day intervals in the farm's own mixing plant. The composition of the feeds is shown in Table I. The only difference between the experimental and the control diet was that the former was supplemented with 15 mmol/kg (0.17%) sodium *n*-butyrate throughout the trial. Sodium *n*-butyrate was obtained by neutralization of technical-grade *n*-butyric acid marketed by Reanal Fine Chemicals Co. It was mixed in the diet in air-dry state, after multiple dilutions and homogenization with corn grits. The piglets were fed the experimental diet from day 7 after transfer to the battery. The amount of feed consumed by the pigs was recorded in each group. The pigs were weighed after transfer to the battery, at the time of grouping, and upon transportation to the abattoir.

Sampling. Processing and analysis of feed samples

The experimental and control diets were sampled on a total of 12 occasions. The dry matter content was determined and the feeds were analysed by Weendei's analytical methods. The deviations of the obtained values from the documented ones were always within the tolerable limits. In the amount mixed in the diet, sodium *n*-butyrate, though hygroscopic, did not affect the dry matter content of the feed mix.

To measure sodium *n*-butyrate concentration, three weight units of distilled water were added to one weight unit of feed. Subsequently butyrate was extracted in a shaker for 30 min (at 60 rpm). After centrifugation at 5000 rpm for 15 min the supernatant was treated with 25% metaphosphoric acid at a ratio of 5 : 1 and stored at —20 °C until used for the gas chromatographic determination.

Blood samples were taken from the pigs at 63, 109 and 206 days of age. As anticoagulant, Na_2EDTA (for blood count and differential blood count) or heparin solution (for urea and enzyme determinations) was used. The plasma was separated from the heparin-treated samples by centrifugation at 5000 rpm for 15 min and it was stored in liquid nitrogen until used for sodium *n*-butyrate and enzyme determinations.

Gastric and intestinal contents and tissue samples were taken immediately after slaughter from the stomach, hindmost portion of the ileum and the caecum. After centrifugation at 5000 rpm for 15 min the supernatant of the gastric and intestinal content was prepared for volatile fatty acid (VFA) determination in the same way as the feed samples were processed for determination of butyrate concentration. For bacteriological examination, ileal and caecal portions were isolated by ligation and stored at —20 °C until processed. On dissection of the digestive organs at the abattoir, the oesophageal part of

the stomach and certain parts of the intestinal tract (ileum and caecum) were fixed in 10% formalin for microscopic examination.

Methods. Blood samples treated with Na_2EDTA as anticoagulant were analysed in an automatic instrument for red blood cell (RBC) count, haemoglobin (Hb) concentration, packed cell volume (PCV), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), and white blood cell (WBC) count. Knowing the lymphocyte count, details of the differential blood count (the ratios of segmented neutrophils, eosinophils, monocytes and lymphocytes) were given numerically.

Urea concentration of the blood plasma samples stored in liquid nitrogen was determined by the Goedecke (Urastrat®) test and their ASAT and AP activity by Roche tests. The concentration of immunoreactive insulin (IRI) was measured with a ^{125}I-insulin AB/PEG radioimmunoassay (RIA) *in vitro* kit (Isotope Research Institute, Hungarian Academy of Sciences, Budapest).

For evaluating the lesions found on the stomach mucosa a score system was established. The mucosal lesions seen in the oesophageal part of the stomach were assigned into one of the following six "severity grades".

1: Physiological status (no pathological lesion)
2: Fine, yellowish coating
3: Rough, rugged crusts
4: Ulcers of small size
5: Large ulcers and small, incipient cicatrices
6: Large ulcers involving the whole oesophageal part of the stomach, and/or extensive cicatrices with oesophagostenosis.

During the histological examination of ileal and caecal sections stained with haematoxylin and eosin, the cells of the intestinal villi and crypts were counted under a light microscope, and their length and depth, respectively, was measured with an ocular micrometer.

Sodium *n*-butyrate and VFA concentrations of the blood plasma were determined by the method of Husvéth and Gaál (1988) and VFA concentration of the gastric, ileal and caecal contents according to the method of Counotte et al. (1979), by gas chromatography.

Bacteriological examination. The bacterial flora of the intestinal content (coliform bacteria, *Streptococcus* spp., *Clostridium* spp., *Lactobacillus* spp.) was determined by surface culture of a dilution series prepared from the intestinal contents, while total viable count was determined by Koch's plate-pouring method.

Biometric evaluation of the results obtained for the control and experimental groups was done by Student's *t* test.

Results

Before starting the trial, the sodium *n*-butyrate to be mixed in the feed was checked for purity and the feeds were examined for *n*-butyrate content. The dry matter content of sodium *n*-butyrate was 91%, and the butyrate content of the preparation was 8.5 mol/kg. The control diets did not contain detectable *n*-butyrate. The average *n*-butyrate content of the experimental diets was 11.1 mmol/kg.

The experimental animals ate the test diets with relish. According to the data shown in Tables II, III and IV, though average daily feed intake was nearly the same in the two groups while on the battery, due to increased feed intake of the experimental group in a later phase of fattening the overall feed consumption of the experimental pigs was by 8.9% higher (Table IV).

The data collected at the farm throughout the trial and summarized in Tables II, III and IV indicate that the experimental pigs showed higher body mass gain throughout. Based upon the weighing performed on day 97 of the fattening period 28 experimental pigs (average net liveweight: 100.4 kg) and 12 control animals (average net liveweight: 100.8 kg) reached the slaughter weight. At the same time, by the end of the feeding trial, all pigs of the experimental group reached the required slaughter weight (48 pigs, average net live-

Table II

Results of feeding the butyrate-containing diet in the 76-day battery rearing period

Indices	Control group	Experimental group
Starting piglet stock (n)	82	82
Starting total liveweight (kg)	574	574
Mean individual body mass at the start of the trial (kg)	7.0	7.0
Mean age at the start of the trial (days)	31	31
Number of deaths in the battery (n)	5	1
Total body mass of dead piglets (kg)	67	10
Number of culled piglets (n)	2	2
Total body mass of culled piglets (kg)	37	28
Mean individual body mass at the end of battery rearing	34.8	37.9
Body mass gain (kg)	2036	2416
Mean individual body mass gain (g/day)	350	402
Mean individual feed consumption (g/day)	1032	997
Amount of feed used up for 1 kg body mass gain (kg)	2.95	2.48
Total number of feeding days	5817	6015
Number of pigs transferred to the fattening house (n)	75	79
Total body mass of pigs transferred to the fattening house (kg)	2610	2990

weight: 99.4 kg). In the control group only 50 pigs reached the required slaughter weight (mean net liveweight: 101.7 kg); 13 animals were retarded in growth (mean net liveweight: 60 kg).

Pigs of the experimental group used up less feed for 1 kg body mass gain (Tables II, III and IV).

Clinical observations. Throughout the 215-day feeding trial, the pigs fed a diet containing sodium *n*-butyrate not only had better appetite but they also developed more rapidly, their skin was brighter, their haircoat smoother, and the experimental group was more uniform. Mass occurrence of diarrhoea or any other disease was not observed in either of the groups. The sporadic deaths recorded during the trial fell to the period of battery rearing in both

Table III

Results of feeding the butyrate-containing diet in the 139-day fattening period*

Indices	Control group	Experimental group
Deaths (n)	—	—
Technological culling (n)	—	3
Body mass of culled pigs (kg)	—	98
Number of pigs sent to the abattoir	62	76
Net liveweight of pigs sent to the abattoir (kg)	6269	7526
Number of retarded (substandard) pigs at end of trial (n)	13	—
Net liveweight of retarded pigs (kg)	780	—
Total body mass gain (kg)	3659	4536
Mean individual body mass gain (g/day)	369	475
Mean individual feed consumption (g/day)	1631	1867
Feed used up for 1 kg of body mass gain	4.42	3.93
Totalnumber of feeding days (n)	9921	9541

* 12 control and 27 experimental pigs were slaughtered on day 97 of fattening, when they reached a liveweight of 100 kg.

Table IV

Overall results of feeding the butyrate-containing diet (at the end of the 215-day period)

Indices	Control group	Experimental group
Total number of feeding days	15,738	15,556
Total body mass gain (kg)	5,695	6,952
Mean individual body mass gain (g/day)	362	447
Mean individual feed consumption (g/day)	1,408	1,533
Amount of feed used up for 1 kg of body mass gain (kg)	3.89	3.43

Table V

Comparison of blood samples collected from pigs of different age

Indices	63 days old		109 days old		206 days old	
	Control (n=7)	Experimental (n=4)	Control (n=12)	Experimental (n=11)	Control (n=10)	Experimental (n=10)
Red blood cell count (RBC, $\times 10^{12}$)	5.76±0.64	5.94±1.11	6.31±0.43	6.62±0.32	6.22±0.32	6.34±0.37
Haemoglobin concentration (Hb, mM)	6.41±0.41	6.34±1.28	6.82±0.48	7.02±0.53	7.02±0.89	7.77±0.46
Packed cell volume (1/1)	0.348±0.031	0.364±0.078	0.364±0.028	0.384±0.032	0.403±0.029	0.392±0.030
Mean corpuscular volume (MCV, fl)	60.40±2.1	60.80±5.30	57.65±2.00	58.10±1.70	64.70±3.10	61.9±2.8
Mean corpuscular haemoglobin (MCH, fM)	1.11±0.04	1.07±0.07	1.08±0.03	1.06±0.09	1.26±0.04	1.23±0.05
Mean corpuscular haemoglobin concentration (MCHC, mM/l)	18.5±0.7	17.6±0.9	18.8±0.5	18.2±0.4	19.5±0.7	19.9±0.8
White blood cell count ($\times 10^9$/l)	16.7±5.1	19.7±3.1	17.9±3.5	18.7±2.8	16.0±4.4	18.9±3.6
Segmented neutrophils ($\times 10^9$/l)	3.6±1.2	3.1±0.8	5.8±2.1	6.3±2.3	5.6±5.0	4.4±1.3
Eosinophils ($\times 10^9$/l)	0.33±0.50	0.44±0.63	0.34±0.35	0.19±0.20	0.14±0.23	0.56±0.47
Monocytes ($\times 10^9$/l)	0.66±0.41	0.95±0.52	0.65±0.32	0.54±0.28	0.67±0.41	0.69±0.36
Lymphocytes ($\times 10^9$/l)	12.1±5.4	15.2±3.4	11.1±2.3	11.7±3.1	8.8±3.7	13.2±3.8

* $\bar{x}\pm SD$

the control (five deaths: two pigs died of gastric ulcer and bleeding, and the other three died of catarrhal enteritis, acute cardiac failure and pulmonary oedema) and the experimental group (one pig died of asphyxia). The animals substantially falling behind the group average were culled: two control and five experimental pigs were sold or slaughtered as technological wastage.

Quantitative and qualitative analysis of blood samples collected at 3 different times during fattening revealed no appreciable difference between the experimental and the control group (Table V). There was no significant difference in blood plasma urea concentration, ASAT and AP activity, which was measured twice (Table VI). The only difference was that the experimental pigs had slightly lower blood plasma urea concentrations at both testing times. The VFA and immunoreactive insulin concentrations of the blood plasma were determined once: they gave higher values in the experimental group (Table VI).

The frequency distribution of lesion scores reflecting the status of mucous membrane in the oesophageal part of the stomach was similar in the two groups (Fig. 1). Stomachs with completely intact mucosa were scarcely found. Ulcerative lesions were diagnosed in 17.7% and 9.2% of the control

Table VI

Urea concentration, ASAT and AP activity, and VFA and immunoreactive insulin concentration of the blood plasma in the control and experimental groups

	109 days old		206 days old	
	Control	Experimental	Control	Experimental
Urea (mg/100 ml)	15.59 ± 1.26 n=11	13.85 ± 1.23 n=8	15.46 ± 2.53 n=12	14.22 ± 1.82 n=16
ASAT (U/l)	24.4 ± 3.5 n=12	23.5 ± 3.1 n=9	36.9 ± 6.8 n=10	37.0 ± 5.2 n=18
AP (U/l)	45.5 ±14.4 n=12	45.2 ±17.5 n=9	41.6 ± 8.3 n=10	42.0 ±14.1 n=18
VFA (μmol/l)			n=3	n=3
Acetic acid	NT	NT	1304.4 ±49.1	1755.6 ±163.8
Propionic acid	NT	NT	9.8 ±1.9	9.3 ±0.4
n-butyric acid	NT	NT	13.9 ±3.8	27.1 ±14.8
Immunoreactive insulin (mU/l)	17.9 ± 5.2 n=9	30.3* ±13.5 n=8	NT	NT

NT = not tested; $\bar{x} \pm SD$; * significant difference from the control on $P < 0.05$ level

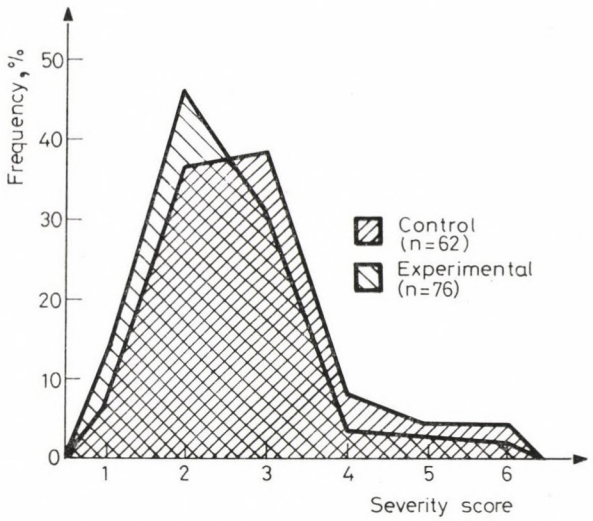

Fig. 1. Frequency distribution (%) of gastric ulcer

and experimental pigs, respectively. Light microscopic examination indicated a substantial increase in the number of cells constituting the ileal microvilli and in the length of the microvilli (33.5% and 30.1%, respectively). A substantial increase in the number of cells forming the crypts and in crypt depth occurred only in the caecum (59.1% and 23.6%, respectively; Table VII).

VFA concentration of the gastric and intestinal content. *n*-butyric acid was detectable only in the gastric content of experimental pigs (Table VIII). The acetic acid, *n*-butyric acid and total VFA concentrations of the ileal

Table VII

Histological examination of ileal and caecal samples taken from pigs slaughtered at 206 days of age

Indices tested	Control		Experimental	
	Ileum (n=5)	Caecum (n=5)	Ileum (n=5)	Caecum (n=5)
Cell count (n)				
Microvillus	29.6±3.4	—	39.5±4.6**	—
Crypt	20.6±2.9	37.4±8.2	26.0±2.8*	59.5±7.5**
Total:	50.5±6.0		65.2±6.2**	
Length (μm)				
Microvillus	234.0±25.5	—	304.4±15.0***	—
Crypt	201.1± 5.9	381.7±38.1	214.9± 9.7^{NS}	471.8±23.0**
Total:	435.1±24.6		519.3±21.3***	

$\bar{x}$±SD. Level of significant difference between respective values of the control and experimental group: NS: not significant; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$

Table VIII

Comparison of gastric ileal and caecal contents for VFA concentration in control and experimental pigs at 206 days of age

VFA (μmol/g)	Control			Experimental		
	Stomach (n=5)	Ileum (n=5)	Caecum (n=5)	Stomach (n=4)	Ileum (n=7)	Caecum (n=7)
Acetic acid	0	16.5±8.4*	38.4±15.3	8.5±4.7	34.9±11.8^{+}	40.1±11.4
Propionic acid	0	2.8±1.7	18.6±9.4	6.9±4.6	4.4±1.6	19.3±5.8
i-butyric acid	0	0.4±0.2	1.2±0.7	0	0.4±0.3	1.6±0.7
n-butyric acid	0	1.8±2.5	5.1±3.2	2.9±0.3	9.0±6.3^{+}	6.0±2.6
i-valerianic acid	0	0.4±0.4	2.1±1.0	0	0.8±0.2	2.6±1.0
n-valerianic acid	0	0	1.7±0.7	0	0	1.8±0.6
Total:	0	21.8±11.3	67.6±28.8		49.5±15.1^{++}	71.4±20.6

* $\bar{x}\pm$SD. Level of significant difference between respective values of the control and experimental group: $^{+}$ $P < 0.05$; $^{++}$ $P < 0.01$

content were significantly higher in the experimental groups. No appreciable difference was found between the two groups in the propionic acid concentration of the ileum and VFA concentration of the caecum either in distribution or in absolute quantity (Table VIII).

Table IX and Fig. 2 show the results of bacteriological examination of intestinal contents collected at slaughter. The percentile proportion of coliforms in the ileal content was higher in the control than in the experimental animals. This can be explained by the fact that in the experimental pigs the coliform count decreased and the counts of *Lactobacillus* spp. rose. The percentile proportion of coliform bacteria in the different intestinal portions was in a significant negative correlation ($r = -0.9656$; $P < 0.05$) with the *n*-

Table IX

Comparison of the ileal and caecal microflora of control and experimental pigs (in samples taken from pigs slaughtered at 241 days of age)

Bacterial count, log/g intestinal content	Control pigs		Experimental pigs	
	Ileum (n=5)	Caecum (n=5)	Ileum (n=5)	Caecum (n=5)
Coliform bacteria	$6.23\pm0.23^{a}\neq$	$6.52\pm0.69^{a}\neq$	$5.46\pm1.25^{a}\neq$	$6.11\pm0.83^{a}\neq$
Streptococcus spp.	$6.62\pm0.61^{a}\S\neq$	$7.28\pm0.58^{a}\neq$	$5.81\pm0.98^{ac}\S$	$6.77\pm0.79^{ac}\S\neq$
Clostridium spp.	$3.08\pm1.07^{b}\neq$	$3.04\pm0.78^{b}\neq$	$2.22\pm0.17^{b}\neq$	$2.02\pm1.38^{b}\neq$
Lactobacillus ssp.	$6.32\pm1.02^{a}\neq$	$7.17\pm0.56^{a}\S\neq$	$6.99\pm0.76^{c}\S\neq$	$7.40\pm0.53^{c}\S$

$\bar{x}\pm$SD
a, b, c Significant difference of at least $P < 0.05$ within column
§, ≠ Significant difference of at least $P < 0.05$ within row

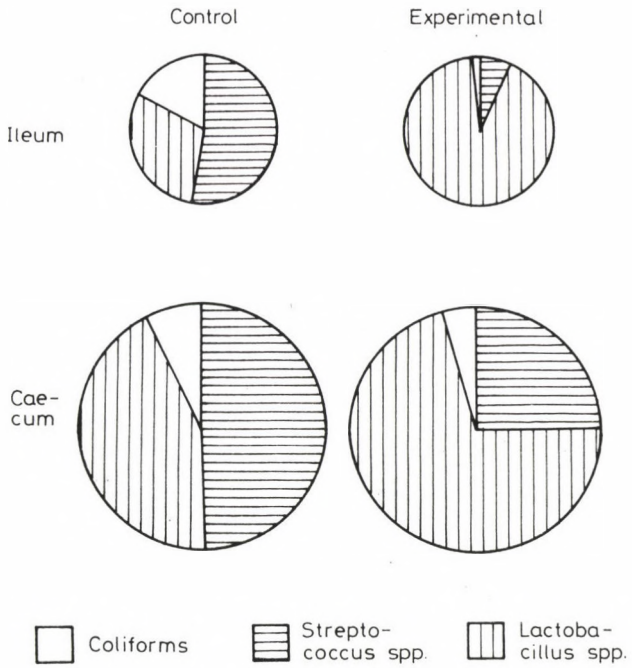

Fig. 2. Percentage distribution of the bacterial flora of the ileum and caecum in the experimental and control groups. Circle size is proportional to total bacterial count (coliforms + + *Streptococcus* spp. + *Lactobacillus* spp.). Control: ileum: 7.9×10^6; caecum: 37.2×10^6; experimental: ileum: 10.7×10^6; caecum: 32.3×10^6 bacteria/g of intestinal content. The percentage of *Clostridium* spp. was always below 0.01%

butyric acid concentration of the intestinal content. There was a negative correlation between the percentile proportion of coliforms and *Lactobacillus* spp. in the ileum ($r = -0.7303$; $P < 0.05$). There was no appreciable difference between the two groups in the other indices tested.

Discussion

In this trial, all economically important indices were more favourable in the experimental than in the control group. The overall results (Table IV) indicated that the average body mass gain of experimental pigs was by 23.5% higher. Though the experimental animals consumed 8.9% more feed, owing to their better feed utilization they used up 11.8% less feed for 1 kg of body mass gain.

Kirchgessner and Roth (1978) and Giesting and Easter (1985) reported on a favourable dietetic effect of citric acid, fumaric acid and propionic acid in pigs. They obtained better results in young animals by feeding organic

Table X

Nutritive effect on pigs of organic acids mixed in different diets

Liveweight (kg)	Organic acid (%)s	Mean individual body mass gain (%)	Mean individual feed consumption (%)	Amount of feed used up for 1 kg body mass gain (%)	Reference
7.5–14.6	Citric acid (2%)	103.2+	96.1	93.2	Giesting and Easter (1985)
18–95	Fumaric acid (0.6%)	103.2	101.3	98.2	Kirchgessner and Roth (1978)
18–95	Fumaric acid (1.8%)	106.7	102.3	95.7	Kirchgessner and Roth (1978)
8–25	Fumaric acid (4%)	103.8	99.3	94.9	Kirchgessner and Roth (1976)
7.5–14.6	Propionic acid (2%)	95.6	89.0	93.1	Giesting and Easter (1985)
7–37.9	Sodium *n*-butyrate (0.17%)	114.9	96.6	84.1	Our own data (battery)
37.9–102	Sodium *n*-butyrate (0.17%)	128.7	114.5	88.9	Our own data (fattening house)
7–102	Sodium *n*-butyrate (0.17%)	123.5	108.9	88.2	Our own data (overall)

+ All production indices are given in % of the control

acids (Table X). We found that young pigs (i.e. those kept in the battery) used up the least feed for 1 kg of body mass gain (Table X). However, the production indices of pigs fed *n*-butyrate remained more favourable also during the fattening period.

According to data of the literature, citric acid, fumaric acid and propionic acid were used for pig feeding in much higher concentrations (0.6 to 4.0%) than sodium *n*-butyrate (0.17%). Our results indicate that sodium *n*-butyrate can exert its effect even in lower concentrations on both prokaryotic and eukaryotic organisms. In our experiments elevation of sodium *n*-butyrate concentration in the ileal content changed the composition of the ileal microflora. It reduced the percentile proportion of coliform bacteria and raised that of *Lactobacillus* spp. in the ileum. By increasing the length of ileal microvilli, sodium *n*-butyrate enlarged the absorptive surface of the ileum, by this means favourably influencing the transport processes. All this resulted in an overall improvement in intestinal digestion, better feed utilization and, as a consequence, much higher body mass gain.

Bugaut (1987) demonstrated that butyrate can be absorbed also from the oesophageal part of the stomach. Possibly this is why only low concentrations of butyrate were present in the gastric content of the experimental pigs.

Acetic acid concentration was also elevated in the stomach and ileum of the experimental pigs, indicating the conversion of butyric acid into acetic acid (Bugaut, 1987). At the same time, butyrate somewhat reduced the severity of ulcerative lesions in the stomach. By elevating the concentration of immunoreactive insulin in the blood plasma it exerted a favourable influence on intermediary metabolism without adversely affecting the clinical parameters examined. Therefore, its effect cannot be considered adverse.

In this trial *n*-butyric acid was used in low concentration, in the form of its sodium salt. Besides its favourable dietetic effect, sodium *n*-butyrate could easily be mixed in the diet. In contrast to other organic acids used by other authors (Table XI) in larger quantities and in acid form, sodium *n*-butyrate did not decrease the pH of the diet (unpublished observations). Thus, its effect was due to its broad biological action rather than to its acidic character.

The biological role of VFA (antimicrobial effect, influence on the mineral and intermediary metabolism, regulation of cell division) has been reported on the basis of numerous *in vivo* experiments (Table XI). In further *in vivo* experiments VFA and their metabolites (3-OH-butyric acid, acetoacetic acid) and other compounds (e.g. succinic acid) participating in intermediary metabolism) were studied for their efficacy (Deutsch et al., 1977; Ghosh et al., 1975; Ginsburg et al., 1973; Griffin et al., 1974; Henneberry and Fishman, 1976; Kruh, 1982; Leder and Leder, 1975). It was found that *n*-butyric acid has much broader biological activity than the other organic acids tested. Through enhanced acetylation of certain histone proteins of eukaryotic organisms it induces gene expression and enhances the production of certain proteins (prekeratin; Gálfi et al., 1985), hormones (chorionic gonadotropin, follicle-stimulating hormone), and enzymes (alkaline phosphatase) (Kruh, 1982). Its effect is highly reversible (Kruh, 1982).

Table XI

Some important biological effects of volatile fatty acids

Effect	Species	Author
–Regulate the growth and ratios of bacteria belonging to the Enterobacteriaceae family	Microorganism	Byrne and Dankert (1979)
– Enhance the intestinal absorption of water and some electrolytes (Na^+, Mg^{++}) and the accumulation of HCO_3^-	Rat, ruminants	Rayssiguier and Remesy (1977)
– Augment the secretion of some hormones (insulin, glucagon)	Rat, ruminants	Montague and Taylor (1968), de Jong (1979)
– Facilitate development of the digestive tract (see ruminant forestomach), increases epithelial cell division and the mass of the intestinal wall (large intestine in rats)	Ruminants, rat	Flatt et al. (1958) Sakata (1987)

The biological effect of *n*-butyric acid is definitely more favourable than that of other organic acids. For this reason, and because of the 9% lower feed costs and the 13% bigger return from sales its use as a growth promoter in pig feeding is justified and can be recommended.

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CHANGES IN THE ELECTRIC RESISTANCE OF THE VAGINAL MUCUS IN OESTROUS SOWS

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Changes in the electric resistance of the vaginal mucus were monitored in a large pig herd at 4-h intervals with a heat detector (Hauptner, Federal Republic of Germany) in multiparous sows (n = 16; littre size = 11.4 ± 2.9) that showed a positive standing test and conceived. The initial value of resistance was 74.4 ± 7.4 ohms. In 36 h this value gradually increased to 93.6 ± 7.6 ohms. One to 2 h after the artificial inseminations (AI) lower resistance values were obtained, though the difference was not statistically significant. In the second part of the experiment 73.9% and 82.7% of the gilts (n = 92) and multiparous sows (n = 98) conceived, respectively, with an average litter size of 9.6 ± 2.2 and 11.0 ± 2.7, respectively. The conception rate of animals that immediately before AI had vaginal mucus resistance values between 75 and 90 ohms was about 20% higher. The only exception were 8 multiparous sows which had an average vaginal mucus resistance of 70 ± 4.6 ohms after the first insemination. Litter size was also the biggest in sows with vaginal mucus resistance between 75 and 90 ohms. This difference was statistically significant for the multiparous sows. The heat detector is considered to be a useful complement to conventional methods of oestrus detection.

Keywords: Vaginal mucus resistance, sow, oestrus detection, heat detector, conception rate, litter size

Detection of the optimum time to inseminate is of fundamental importance for profitable large-scale pig production.

Ovulation takes place 36 to 40 h after the onset of oestrus and lasts 3.8 h on the average. The ova released into the oviducts remain viable for about 12 h, while the spermatozoa maintain their viability for 18–24 h after a 2- to 4-h maturation process. The highest conception rate can be achieved if the sows are inseminated about 12 h before the expected time of ovulation that is 24 to 36 h before the beginning of oestrus proper (onset of the standing reflex) (Hughes and Varley, 1980).

The beginning of oestrus proper and, thus, the optimal time to inseminate cannot be reliably detected in large pig herds where oestrous sows are selected twice a day. This statement is supported by Hungarian studies according to which in the majority of sows the beginning of oestrus proper falls between 4 and 10 a.m. or 7 and 10 p.m. At night, and even more so in the daytime, animals newly coming into oestrus are found only exceptionally (Becze, 1981). This indicates that there is an about 12-h time lag between the onset and the detection of oestrus proper.

Detection of the optimum time to inseminate is rendered difficult by the fact that only 75% of the oestrus sows display oedema and reddening of the vulva, and that in about 40% of them the standing reflex cannot be elicited in the absence of a boar. Therefore, the use of teaser boars is yet indispensable at large-scale pig farms. If teaser boars are not available, the boar spray can be used with good results: this spray contains pheromones and helps to elicit the standing reflex (Hughes and Varley, 1980; Reed, 1984).

The purpose of this study was to elucidate the relationship existing between the electric resistance of the vaginal mucus measured before artificial insemination (AI) and the conception rate.

Materials and methods

The experiments were carried out at a large pig farm where cross-breeding of Hungarian and English Large White pigs with pigs of the Dutch, Swedish and Hungarian Landrace breeds is being conducted. In the period between 18 March and 21 May 1988, 100 gilts and 100 multiparous sows were tested for oestrus in the morning (between 7:30 and 8:30 a.m.) with the help of teaser boars. On the day of selection from 3 o'clock p.m., then next day from 10 o'clock a.m., before artificial insemination (AI) the electric resistance of the vaginal mucus of oestrus sows was measured with a heat detector (Hauptner, Solingen, Federal Republic of Germany) as specified by the manufacturer. AI was done in the presence of a teaser boar, using boar semen ($8–10 \times 10^9$ spermatozoa) freshly diluted in modified Kiev diluent, with a Melrose catheter.

In addition, changes in the electric resistance of the vaginal mucus of 16 multiparous sows with positive standing test were measured at 4-h intervals for 36 h after their selection. The pregnancy of the inseminated sows was determined on the basis of their farrowing. After calculating the mean and standard deviation, our data were evaluated by analysis of variance.

Results

Changes in the electric resistance of the vaginal mucus of 16 multiparous pregnant sows were measured at 4-h intervals after the onset of the standing reflex and are shown in Fig. 1. The initial value of 74.4 ± 7.4 ohms gradually rose to 93.6 ± 7.6 ohms in 36 h. The mean values calculated in the 28th, 32nd and 36th h are, however, only of informatory value, as the values above 100 ohms, measured in 2, 5 and 6 sows at those times, were uniformly taken as 100.

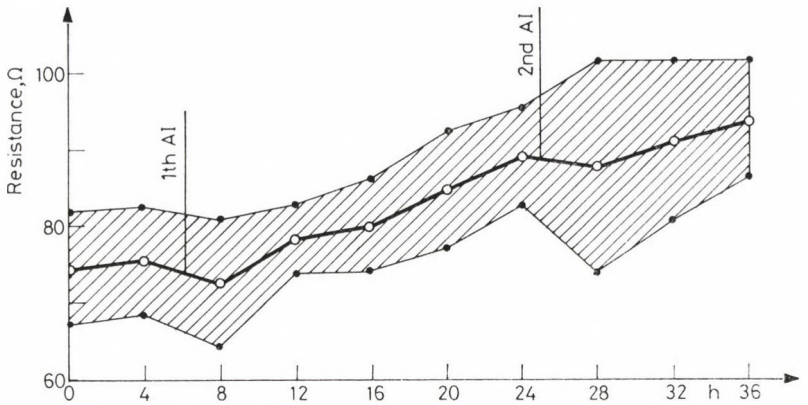

Fig. 1. Electric resistance of the vaginal mucus of multiparous pregnant sows (n = 16) in a 36-hour period following the onset of the standing reflex

The electric resistance of the vaginal mucus tended to decrease in both cases after AI. This decrease was not statistically significant.

The electric resistance of the vaginal mucus, the conception rate, and the litter size obtained for the 100 gilts and 100 multiparous sows (being at their 2nd to 6th parity) are shown in Tables I and II. The data of only 190 out of the 200 pigs were processed as 8 gilts and 2 sows had to be culled. Of the gilts, 73.9% conceived, 16 (17.4%) returned to oestrus, and 8 (8.7%) though did not return to oestrus, remained open. Conception rate was 82.7% for the sows. Twelve sows (12.2%) returned to oestrus and 5 (5.1%), though did not return to oestrus, remained open.

According to the manufacturer's specifications, a vaginal mucus resistance between 75 and 85 ohms is optimal for AI. On the first insemination

Table I

Electric resistance of the vaginal mucus in gilts

Electric resistance of the vaginal mucus (ohms)	First insemination				Second insemination				Litter size	
	Conceived		Returned to oestrus	Open	Conceived		Returned to oestrus	Open	First insemination	Second insemination
	n	(%)	n	n	n	(%)	n	n	($\bar{x}\pm$SD)	($\bar{x}\pm$SD)
60–74	5	(62.5)	1	2	1	(50.0)	—	1	8.4±2.0	—
75–85	24	(80.0)	3	3	3	(60.0)	—	2	9.8±2.9	—
86–90	15	(88.2)	2	—	11	(68.8)	4	1	9.4±2.4	10.0±2.3
91–100	5	(62.5)	3	—	22	(84.6)	3	1	9.6±1.1	9.6±2.7
>100	19	(62.5)	7	3	31	(72.1)	9	3	9.7±1.3	9.4±1.9
Total:	68	(73.9)	16 (17.4)	8 (8.7)	68	(73.9)	16 (17.4)	8 (8.7)	9.6±2.2	

Table II

Electric resistance of the vaginal mucus in multiparous sows

Electric resistante of the vaginal mucus (ohms)	First insemination				Second insemination				Litter size	
	Conceived		Returned to oestrus	Open	Conceived		Returned to oestrus	Open	First insemination	Second insemination
	n	(%)	n	n	n	(%)	n	n	($\bar{x}\pm$SD)	($\bar{x}\pm$SD)
60–74	8	(80.0)	2	—	—		1	—	9.8 ± 2.2^{a}	—
75–85	41	(89.1)	2	3	11	(78.6)	3	—	11.8 ± 2.7^{ab}	9.9 ± 2.6
86–90	18	(85.7)	2	1	25	(96.2)	—	1	10.3 ± 2.0	11.1 ± 2.9
91–100	11	(68.8)	5	—	23	(92.0)	3	2	9.4 ± 3.0^{b}	11.2 ± 2.7
>100	3	(60.0)	1	1	22	(75.9)	5	2	—	11.0 ± 2.6
Total:	81	(82.7)	12 (12.2)	5 (5.1)	81	(82.7)	12 (12.2)	5 (5.1)	11.0 ± 2.7	

[a] $P < 0.05$; [b] $P < 0.01$

32.6% of the gilts and 46.9% of the multiparous sows fell in this range. It was found that the conception rate for gilts and sows inseminated at a time when the electric resistance of their vaginal mucus was between 75 and 90 ohms was about 20% higher than for the remainder. The only exception were 8 multiparous sows which before the first insemination had vaginal mucus resistance values between 60 and 74 ohms (mean: 70.0 ± 4.7 ohms). Litter size was also the biggest in sows with vaginal mucus resistance between 75 and 90 ohms before the first insemination. The difference between various groups of multiparous sows reached the statistically significant level.

Conception rate to the second insemination was the highest in gilts inseminated with vaginal mucus resistance between 91 and 100 ohms and in sows inseminated with values between 86 and 100 ohms. No appreciable difference was demonstrable in respect of the litter size.

Discussion

During heat, and primarily in the period before ovulation, the quantity of vaginal mucus gradually increases and electrolytes, mainly sodium, are increasingly secreted into the cervical and vaginal mucus (Aizinbudas and Dovil'tis, 1962; Feldman et al., 1978).

Changes in the electrolyte concentration of the vaginal mucus during heat allow us to measure the electric resistance and conductivity of the vaginal mucus with an ohmmeter and conductivity meter, respectively. Electrical conductivity is the reciprocal of electric resistance.

Johnson et al. (1982) did not find appreciable differences between oestrous sows inseminated at a fixed time (32 to 34 h after the onset of heat) and those inseminated at a time selected on the basis of vaginal mucus resistance measured with an ohmmeter. Significant differences both in conception rate and in litter size were demonstrated between sows inseminated with freshly diluted semen and with deep-frozen boar semen.

Zink and Diehl (1984), who measured electrical conductivity of the vaginal mucus of sows, obtained a high degree of variation among individuals. They found that a 10% increase in vaginal conductivity provided a reliable basis for determining the optimum time to inseminate swine. However, there was no appreciable advantage associated with the use of vaginal conductivity over the conventional methods.

Harbison et al. (1987) inseminated oestrous sows with fresh or deep-frozen semen in the 24th h after the onset of heat or at a time determined on the basis of vaginal mucus conductivity. They obtained significantly lower conception rates in sows inseminated with deep-frozen semen. At the same time, no appreciable difference was found between sows inseminated at a fixed time or at a time determined on the basis of vaginal mucus conductivity. They called attention to the fact that some sows may show optimal conductivity values even 24 h later. Therefore, when using deep-frozen boar semen, the determination of vaginal mucus conductivity is unsuitable for detecting the optimum time to breed sows.

In our experiments conducted under field conditions, in herds where sows are tested for heat twice a day and those showing a positive standing test are inseminated with freshly diluted semen twice, at an interval of about 7 to 19 h, the Hauptner heat detector was found to be suitable for detecting the optimum time to inseminate. Namely, gilts and multiparous sows inseminated at a time when their vaginal mucus resistance was between 75 and 90 ohms showed an about 20% higher conception rate as compared to the remainder. The only exception were 8 sows in which a mean resistance of 70.0 ± 4.6 ohms was measured immediately after the first insemination. Litter size was also the biggest in sows with vaginal mucus resistance between 75 and 90 ohms. In the multiparous sows this difference reached the statistically significant level.

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EPIDEMIOLOGY OF *SALMONELLA DERBY* STRAINS ISOLATED FROM SWINE, PORK AND PORK PRODUCTS

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A total of 35 *Salmonella derby* strains, isolated from 6 types of samples of porcine origin from 9 different places in Hungary were examined for their characteristics. Thirty-two strains (91%) were of phage type 25, 2 (6%) of phage type 15 and 1 (3%) of phage type 58. Colicin production was observed in 3 (9%) strains. Five strains (14%) were found to be resistant to tetracycline (Tc). The strains harboured plasmids of 2.2, 2.4, 3.4, 4.2 and 72 Md. The 72 Md plasmid appears to be characteristic of *S. derby* and possibly encodes Tc resistance. The 72 Md plasmid belonged partly to incompatibility (Inc) group I_1, while the other plasmid of the same size belonged to Inc. group B. The findings suggest that healthy salmonella carrier pigs carried the infection from the farm to the abattoir. Slaughtering of infected pigs may have led to contamination of the carcasses and, thereafter, that of the pork and pork products.

Keywords: *Salmonella derby*, characteristics, epidemiology, pig, pork, pork product

Salmonella derby was first isolated in Great Britain from human beings who had become ill after consumption of pork pies (Peckham, 1923). Thereafter its isolation from different animal species, food, feed, and environmental samples has been reported by several researchers. *S. derby* was found to be the most predominant serotype in healthy slaughter pigs in the United States of America (Mackel et al., 1965; Keteran et al., 1982; Currier et al., 1986), in Singapore (Liow and Loh, 1977), in Hong Kong (Lo et al., 1967; Chau et al., 1977) and in Egypt (El-Nawawi et al., 1982).

In Hungary, Takács and Nagy (1973*a* and *b*) stated that between 1947 and 1973 *S. derby* was frequently isolated from animals and animal products. In 1986 and 1987 it was found to be the 9th resp. 7th most frequently occurring serotype in human beings (National Institute of Hygiene, 1988). *S. derby* is the predominant serotype in some pig herds (Bíró et al., 1989) and in healthy abattoir pigs (Jayarao et al., 1989). Very scanty literature exists concerning epidemiological studies related to *S. derby*. These facts prompted us to study the characteristics of *S. derby* isolates in order to understand the epidemiological features of *S. derby*.

Materials and methods

S. derby strains

A total of 35 *S. derby* isolates were examined. These isolates consisted of 20 strains isolated from pig faeces: 11 isolates were of farm origin and 9 came from an abattoir. Seven and 6 strains were isolated from pork and pork product samples, respectively. One strain was isolated from a pig carcass (hygiene swab) and another one from clinical material from a pig (Table II). The *S. derby* strains isolated from some swine herds in the Szolnok district (Bíró et al., 1989) and from healthy abattoir pigs in Budapest (Jayarao et al., 1989) were from an earlier part of the study and had been isolated between September 1987 and June 1988. The other strains were obtained from the Salmonella Diagnostic Laboratory, Veterinary and Food Control Service, Budapest. These strains were isolated in June 1988 by regional Veterinary and Food Control Stations.

Phage typing, test for colicin production, antibiotic susceptibility tests, plasmid profile analysis, antibiotic resistance transfer studies, and incompatibility tests were performed at the Phage Department of the National Institute of Hygiene, Budapest.

Phage typing

Phage typing of *S. derby* strains was performed using the phage set elaborated for *S. saintpaul* as described by László (1983). The phage types were designated by Farmer's (1970) mnemonic method.

Determination of colicin type

Colicin typing was carried out according to the method of De Alwis and Thomlinson (1973).

Plasmid profile analysis

Plasmids were detected by the method described by Kado and Liu (1981). The agarose gel electrophoresis was performed as described by Meyers et al. (1976).

Antibiotic susceptibilities and detection of conjugative R plasmids

Mueller–Hinton agar was used for the antibiotic susceptibility tests. Nutrient broth was used for conjugative experiments. Brain Heart Infusion (BHI) agar was used as a basal medium for selective plates with tetracycline (Tc). Antibiotic susceptibility testing of *S. derby* strains was carried out

Table I

Reference R plasmids used in the study

No.	Reference R plasmid	Source	Incompatibility group	Resistant to
1	pIP512	Institut Pasteur	FI	Sm, Cm, Ap
2	pIP112	Institut Pasteur	J1	Km
3	pIP72	Institut Pasteur	B-O	Km
4	R1	N. Datta	F II	Cm, Ap
5	R471a	N. Datta	L-M	A0
6	R40a	N. Datta	C	Ap, Km

Sm = streptomycin; Cm = chloramphenicol; Km = kanamycin; Ap = ampicillin

employing standard antibiotic disks produced by the HUMAN Institute for Serobacteriological Production and Research, Budapest. The strains were examined for their resistance/sensitivity to ampicillin, chloramphenicol, kanamycin, streptomycin, tetracycline, gentamicin, furazolidone, nalidixic acid, neomycin, colistin, polymyxin B and sulphathiazole. The test was done as described by Bauer et al. (1966). Plasmids were detected by the procedures described by Anderson and Lewis (1965*a* and *b*). *E. coli* K 12 J5-3 (rifampicin resistant) was used as recipient.

Incompatibility tests

The compatibility of R plasmids derived from salmonella isolates was examined by the method described by Datta (1977). The reference R plasmids used in the study are shown in Table I.

E. coli transconjugant was used as recipient and incompatibility (Inc) reference plasmid carried in *E. coli* was used as donor.

Results

Thirty-five *S. derby* strains were examined. Thirty-two strains (91%) were of phage type 25, 2 (6%) of phage type 15, while 1 strain (3%) was of phage type 58 (Table II). Three strains (9%) produced colicin, and 5 strains (14%) were resistant to Tc. All examined strains were found to carry 1 to 5 plasmids. Sixteen (46%), 7 (20%), 5 (14%), 5 (14%) and 2 (6%) strains carried 1, 2, 3, 4, and 5 plasmids, respectively. A 2.2 Md plasmid was found to co-exist along with a 72 Md plasmid, while the 2.4 and 3.4 Md plasmids, although found to occur with the 72 Md plasmid, were also found to occur independently of the 72 Md plasmid. A 4.2 Md plasmid was seen to occur

Table II

The characteristics of the *Salmonella derby* isolates

Sample	No. of isolates	Strain no.	Phage type	Colicin production	Resistance pattern	Plasmid profile Md				
P.f.(F)	2	1,2	25	—	—	2.4				
P.f.(A)	1	3	25	—	—	2.4				
P.f.(F)	4	4–7	25	—	—		3.4			
P.f.(A)	1	8	25	—	—		3.4			
P.f.(F)	2	9, 10	15	—	—		3.4			
Pork	1	11	25	—	—		3.4			
Pork	2	12, 13	25	—	—		3.4			
Pork p.	2	14, 15	25	—	—		3.4			
Clinical (pig)	1	16	25	—	Tc					72
P.f.(F)	1	17	58	+	—	2.2				72
P.f.(A)	2	18, 19	25	—	—	2.2				72
Pork	1	20	25	—	Tc	2.2				72
Pork	1	21	25	—	—	2.2				72
Pig (HS)	1	22	25	—	Tc	2.2				72
Pork p.	1	23	25	—	—	2.2				72
P.f.(F)	1	24	25	+	—	2.2	2.4			72
P.f.(A)	2	25, 26	25	—	—	2.2	2.4			72
Pork	1	27	25	—	—	2.2	2.4			72
P.f.(A)	1	28	25	+	—	2.2		3.4		72
P.f.(A)	3	29–31	25	—	—	2.2	2.4	3.4		72
Pork p.	1	32	25	—	—	2.2	2.4	3.4		72
Pork p.	1	33	25	—	—	2.2	2.4	3.4		72
Pork	1	34	25	—	Tc	2.2	2.4	3.4	4.2	72
Pork p.	1	35	25	—	Tc	2.2	2.4	3.4	4.2	72

P.f.(F) = pig faeces of farm origin; P.f.(A) = pig faeces of abattoir origin; pig (HS) = = pig carcass swabs; Tc = tetracycline; Pork p. = pork products

in two strains which contained 5 plasmids each. The 2.2 and 72 Md plasmids were found in all the strains, except some of the strains which carried only one plasmid of 2.4 or 3.4 Md. Strains which were colicin producing or Tc resistant, contained the 72 Md plasmid. The 35 strains belonged to 8 different plasmid profile types. The distribution of 6 plasmid profile types occurring in different types of samples is shown in Table III.

The five Tc-resistant strains were examined for their ability to transfer their antibiotic resistance to a recipient *E. coli* K 12 strain (Table IV). All

Table III

Distribution of plasmid patterns and origin of the samples

Plasmid profile pattern (Md)	Faeces		Pork	Pork product
	Farm	Abattoir		
2.4	Szolnok ⟶	Budapest		
3.4		Baja ⟶	Baja	
2.2, 72	Szolnok ⟶	Budapest		
2.2, 2.4, 72	Szolnok ⟶	Budapest		
2.2, 2.4, 3.4, 72		Budapest ⟶	Budapest	
2.2, 2.4, 3.4, 4.2, 72			Szekszárd ⟶	Szekszárd

The arrows indicate the probable transfer of *Salmonella derby* strain with the same plasmid profile type from one place to another or from one sample to another

Table IV

Conjugative R plasmids and their incompatibility groups

Donor			Recipient		
Sample	RP	Plasmids (Md)	RP transfer	Plasmids (Md)	Inc. group
Clinical (pig)	Tc	72	Tc	72	I_1
Pork	Tc	2.2, 72	Tc	2.2, 72	I_1
Pork	Tc	2.2, 72	Tc	2.2, 72	I_1
Pork	Tc	2.2, 2.4, 3.4, 4.2, 72	Tc	2.2, 2.4, 4.2, 72	B
Pork p.	Tc	2.2, 2.4, 3.4, 4.2, 72	Tc	2.2, 2.4, 4.2, 72	B

RP = resistance profile; Inc. group = incompatibility group; Tc = tetracycline

the five strains could transfer their Tc resistance to the recipients. The donors and the transconjugants were examined for their plasmid profiles. In strains 16, 20, and 22 the plasmid profiles of the donors and the transconjugants were the same. The 72 Md plasmid in these strains belonged to Inc. group I_1, while in strains 34 and 35 all plasmids except a 3.4 Md plasmid were transferred to the transconjugants. The 72 Md plasmid in these strains belonged to Inc. group B.

Discussion

The results indicate that phage type 25 was the predominant type among the *S. derby* strains examined. No literature was available about phage types of *S. derby*. László (1988) elaborated a phage typing scheme for *S. saintpaul* and *S. derby*. She identified four different phage types, viz. 11 (30%), 15

(12%), 21 (8%) and 25 (48%) from human isolates. Phage type 25 which was found in human beings was observed to occur in all the porcine samples and in all the 9 places where the strains came from.

Colicin production was observed in 3 strains (8%). This is in good agreement with the figures reported: 2.5% (Simmons et al., 1988), 2% (Sharma et al., 1984) and 10% (Frederciq, 1957). Production of colicin in salmonella species in the intestinal tract results in suppression of the resident flora and increased ability to survive in blood, peritoneal fluid and alimentary tract of the infected animal (Elwell and Shipley, 1980). In addition, colicinogeny may occur in association with drug resistance and is often co-transferable with drug resistance plasmids (Sharma et al., 1984).

With the exception of a 4.2 Md plasmid, other plasmids of 2.2, 2.4, 3.4, and 72 Md were found to be common in the 35 strains examined. All the five Tc-resistant strains could transfer their resistance to *E. coli* K 12 recipients. The 72 Md plasmid was present in all these five Tc-resistant strains. This large plasmid appears to be an R plasmid probably encoding for Tc-resistance. In 3 strains the 72 Md plasmid belonged to Inc. group I_1, while in the other 2 strains plasmids of the same size belonged to Inc. group B. The 72 Md plasmid was also found to be present in non-colicinogenic and antibiotic-sensitive strains. This plasmid can perhaps be said to be characteristic of *S. derby* strains. In other serotypes, e.g. in *S. dublin* (Baird et al., 1985), *S. enteritidis* (Nakamura et al., 1985) and *S. typhimurium* (Helmuth et al., 1985) serotype-specific plasmids have been recognized. The contribution of plasmids to clinical and economic problems associated with salmonellas is well recognized (Terakado et al., 1983). Eight plasmid profile types were observed. In the Szolnok district alone 4 different plasmid profile types were seen to occur in pig faeces from different farms. This suggested that different clones of *S. derby* might exist.

Three out of the 4 plasmid profile types of *S. derby* which were prevalent in some of the swine herds in the Szolnok district were also found to occur in the *S. derby* isolates obtained from pig faeces from an abattoir in Budapest. This indicated that healthy slaughter pigs, which had been infected on the farm itself, may have carried the infection to the abattoir. Similar observations were made by Williams and Newell (1970) who found that salmonella carrier pigs leaving the farm were the primary source of contamination at the abattoir. Two strains of *S. derby* (Baja abattoir), one of which was from pig faeces and the other from a pork sample, carried a plasmid of 3.4 Md. Morgan et al. (1987) found that the carcass contamination of healthy slaughter pigs was primarily due to intestinal salmonella infection. Moo et al. (1980) stated that when lymph nodes from healthy salmonella carrier pigs are incised during the normal procedure of meat inspection, a substantial reservoir of salmonella can be exposed and transferred to other parts of the carcass via

infected implements or personnel. Another two strains (Szekszárd), one of which was isolated from a pork sample and the other from a pork product, were of the same plasmid profile type and resistant to tetracycline. The infected carcass, when utilized for preparation of pork products, could possibly have resulted in contaminated products.

The study reveals the probable epidemiological links concerning the mode of transfer of *S. derby* strains from the farm to the abattoir and from pork to pork products.

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PATHOLOGICAL AND IMMUNOLOGICAL STUDY OF GOOSE EMBRYOS EXPERIMENTALLY INFECTED WITH DUCK PLAGUE VIRUS

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A total of 240 embryonated goose eggs obtained from two susceptible flocks were used. Half of the eggs were inoculated into the allantoic cavity with a virulent strain (7593) of duck plague virus isolated from an acute outbreak, and the other half were inoculated with the attenuated vaccine virus (KAPEVAC). Ten, 100 or 1000 CPU/0.1 ml virus were given on days 12 and 20 of incubation. Embryos that died and surviving embryos killed at 5-day intervals were examined by light and electron microscopy. The yolk and the serum of embryos that survived until hatching were assayed for antibody content. Lymphocytes separated from the blood were used for the immunorosette formation and lymphocyte stimulation tests.

Pathomorphological changes indicative of virus replication occurred in the liver, kidney, myocardium, gizzard muscle and chorioallantoic membrane (CAM) of the embryos in the case of both virus strains. The time of onset and severity of these changes and the time and rate of embryonic mortality depended on the virulence of the strain used for inoculation, the virus dose and the time of inoculation.

Virus-neutralizing (VN) antibodies were demonstrable neither in the yolk nor in the serum of goslings exsanguinated after hatching. The lymphocytes recognized the virus antigen in the *in vitro* cellular tests and responded to it with blastogenic transformation.

As opposed to adult birds, in the embryos duck plague virus infection did not cause damage to the digestive tract mucosa and the lymphoid organs.

Keywords: Duck plague, virus, goose embryo, pathology, immunology, experimental infection

Duck plague (DP) is a viral disease affecting certain waterfowl species (domestic and wild ducks, geese and swans). It was first described in ducks by Baudet (1923) in the Netherlands. Subsequently it was reported from other countries of Europe, from Asia and North America (Jansen, 1968; Leibovitz, 1968; Newcomb, 1968; Friend and Pearson, 1973; Hofstad et al., 1978; Montgomery et al., 1980). On the basis of its morphological and biological properties, the causative agent was assigned to the Herpesviridae family (Breese and Dardiri, 1968). The pathomorphological changes are most expressed in the digestive tract and liver both in ducks and in geese. In the mucous membrane of the digestive tract there are haemorrhages, swelling or ulcerative inflammation of the lymph follicles, superficial necrosis, and formation of croupous or diphtheroid pseudomembranes. Dystrophy, haemorrhages and necrosis can

be seen in the liver, accompanied by signs of circulatory disturbance (hyperaemia, oedema) and haemorrhages in other organs. The lymphoid tissues are characterized by lymphocytic degeneration and necrosis, and the mucosal epithelial cells of the digestive tract and the hepatocytes contain intranuclear inclusion bodies (Jansen, 1968; Leibovitz, 1969; Friend and Pearson, 1973; Hofstad et al., 1978; Montgomery et al., 1980; Vetési et al., 1982). The appearance and severity of the gross pathological lesions greatly vary by the virulence of the causative virus, and depend on the age, breed and immunobiological status of the birds.

Contrary to the pathomorphological lesions seen in adult birds, changes developing in the embryos are little known. Duck and goose embryos inoculated with duck plague virus on day 9 or 10 of incubation die 5 to 10 days after the inoculation. At necropsy these embryos show diffuse oedema, punctiform haemorrhages, liver dystrophy, and oedema and haemorrhages in the chorioallantoic membrane (CAM) and yolk sac wall (Jansen, 1968; Dardiri, 1975; Hofstad et al., 1978; Vetési et al., 1982). Mortality was directly proportional to the virulence of the virus strain.

Though the virus may persist in the cloaca for a long time, attempts to isolate it from embryonated eggs or newly hatched birds have consistently failed until quite recently; thus, spread of DPV via the germinative route could not be proved. Recently, however, the possibility of germinative infection has been proved by infection experiments in Pekinese and muscovy ducks as well as in mallards. It is supposed that the infection of consecutive generations of free-living mallards can also be traced back to germinative infection (Burgess et al., 1979; Montgomery et al., 1980).

Only one serotype of the causative herpesvirus is known. However, DPV strains may widely vary in virulence (Dardiri and Gailiunas, 1969; Dardiri, 1975). The virus is similar to other herpesviruses also in its biological properties. Its pathogenicity is species dependent. Inapparent infection is common. The virulent virus was isolated from waterfowl with high titres of VN antibodies. Similarly to other herpesviruses, numerous aspects of virus carriership are still unclear (Erickson et al., 1974; Dardiri, 1975). Seroconversion is not pronounced. In vaccinated waterfowl the log 10 titre of VN antibodies was around 1.75. After infection with the virulent virus this titre rose to 4 log 10 and persisted on that level for several months. Layers having antibodies of such titre conferred passive maternal immunity on their offspring. However, the maternal antibody levels rapidly declined and the young birds succumbed to virulent virus infection performed at 13 days of age (Erickson et al., 1974; Dardiri, 1975).

Some observations, however, suggest that adult waterfowl, which have been vaccinated or recovered from virulent virus infection but already lack serum antibodies, may prove to be protected against duck plague virus (DPV).

This fact suggests the possible role of cell-mediated immunity and local immunity of the mucous membranes (Dardiri, 1975).

The purpose of this experiment was to study the pathomorphological lesions and immune response elicited in goose embryos by DPV strains of varying virulence.

Materials and methods

Birds

A total of 240 preincubated goose eggs obtained from two different flocks (I and II) were used (120 eggs from each flock). According to the virological and serological examination, flock I was free from parvovirus, herpesvirus, adenovirus and mycoplasma infection. Flock II was a clinically healthy, large flock. Samples taken from the eggs were checked for bacterial and fungal infections by culturing on agar plates.

Inoculation and sampling

Half of the embryos were inoculated with DPV strain 7593, a virulent strain isolated from an acute outbreak in our institute in 1978. The other half was inoculated with an attenuated strain used for vaccine (KAPEVAC) production. Inoculation was done as shown in Table I. In both groups, half of the embryos came from flock I and the other half was obtained from flock II. Embryos from the two flocks were incubated separately.

The eggs were candled daily until the embryos hatched on day 28 to 30 of incubation.

Five embryos were killed at 5-day intervals. These embryos, together with those that died, were necropsied and their organs were studied by light

Table I

Inoculation of the embryos

Group	Number of embryos	Time of inoculation (day)	Treatment
A	60	12	Virulent virus (10, 100 and 1000 CPU in 0.1 ml PBS) into the allantoic cavity of 20 eggs each
B	40	20	Virulent virus (10 and 1000 CPU in 0.1 ml PBS) into the allantoic cavity of 20 eggs each
C	60	12	Attenuated virus (10, 100 and 1000 CPU in 0.1 ml PBS) into the allantoic cavity of 20 eggs each
D	40	20	Attenuated virus (10 and 1000 CPU in 0.1 ml (PBS) into the allantoic cavity of 20 eggs each
PC	20	12	0.1 ml PBS into the allantoic cavity
C	20	—	—

and electron microscopy. Virus reisolation was attempted from some organs. The yolk and the serum of birds surviving until hatching were assayed for specific antibody content. Lymphocytes separated from the blood were used in the lymphocyte stimulation and immunorosette formation test.

The whole body of the embryos was used for *light microscopy*. Samples for electron microscopy were taken from the liver, heart, gizzard wall and CAM of two group A embryos each killed on postinoculation day 5 and 10, respectively, as described earlier (Glávits et al., 1984).

Immunological tests

The *lymphocyte stimulation* and *immunorosette formation tests* were done as described earlier (Glávits et al., 1984), using blood samples from hatched birds. The same blood samples were used for *determination of antibody titres* by the VN test.

The presence of specific VN antibodies of maternal origin in the yolk of 5 noninfected (control) embryos each from flocks I and II was also checked by the VN test.

Virological examination

The livers of embryos killed at 5-day intervals and those of embryos that died, were pooled by group and time and used for virus isolation.

Results

The mortality found for groups A, B, C and D is shown in Figs 1 and 2. Two deaths each occurred in groups PC and group C. The mortality rate was 100% and 40% in the group inoculated with virulent virus on incubation day 12 and 20, respectively. In the groups inoculated with the attenuated strain on day 12 and 20 of incubation the mortality rate was 70 and 25%, respectively. With both virus strains, the majority of deaths occurred among embryos inoculated with the larger virus dose, and embryos surviving until hatching were mainly those which had been inoculated with the lower virus dose.

In the case of both virus strains, *necropsy* of embryos that had died at an early stage of incubation revealed oedema and haemoglobinous infiltration of the subcutaneous connective tissue and muscles (Fig. 3), as well as punctiform haemorrhages on the CAM, in the subcutaneous connective tissue and muscles, and under the serous membranes. In embryos that had died of infection at a later stage of incubation there was hepatitis accompanied by formation of necrotic foci, liver dystrophy, signs of icterus, occasionally myocarditis

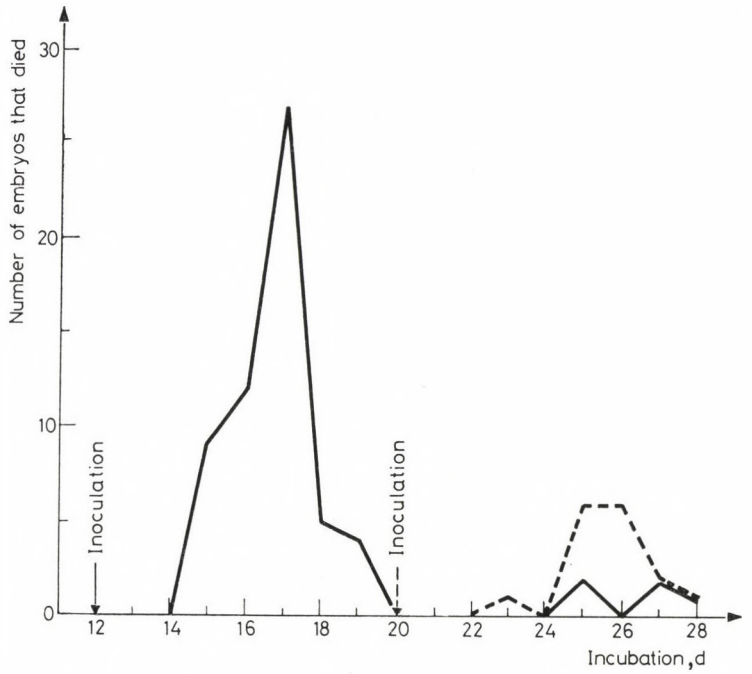

Fig. 1. Mortality of goose embryos inoculated with virulent virus on day 12 (group A, solid line) and day 20 (group B, broken line)

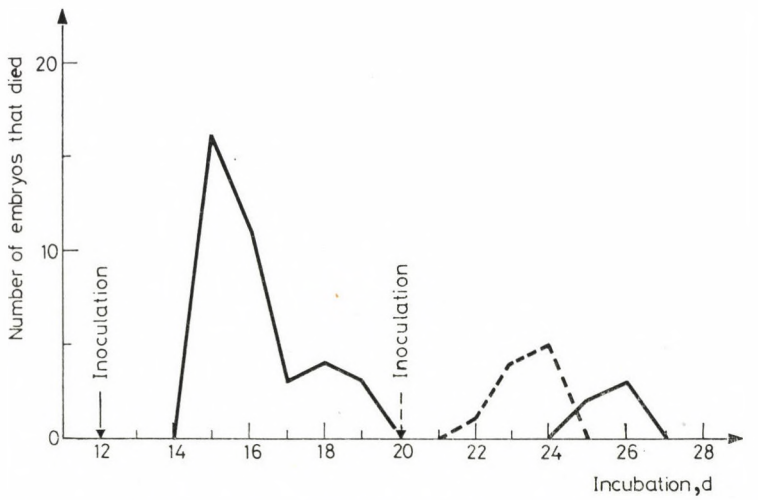

Fig. 2. Mortality of goose embryos inoculated with attenuated virus on day 12 (group C, solid line) and day 20 (group D, broken line)

Table II

Pathomorphological changes in goose embryos inoculated with duck plague virus strains of different virulence

Group of embryos (see Table I)	Pathomorphological lesions	
	on PI day 4–5	on PI day 7–8
A	– oedema and haemoglobinous infiltration of the CAM, skin and muscles,	– serous, in places fibrinous, inflammation of the CAM
	– haemorrhages in the CAM, liver and heart	– hepatitis with focal necroses of the parenchyma, infiltration with heterophilic granulocytes, icterus
	– serous hepatitis	
	– acidophilic intranuclear inclusion bodies in the liver, kidney, myocardium and gizzard muscle	– necroses in the myocardium and gizzard muscle
C	Lesions similar to, but milder than, those found for group A	
B	– serous inflammation of the CAM	– retardation in development (failure to hatch)
	– hepatitis with interstitial infiltration by heterophilic granulocytes, icterus, intranuclear inclusion bodies	– serous inflammation of the CAM with focal necroses
		– hepatitis with interstitial inflammation by lymphocytes and histiocytes
	– acidophilic intranuclear inclusions in the kidneys, myocardium and gizzard muscle	– necroses in the myocardium and gizzard muscle lympho-histiocytic epicarditis
D	Lesions similar to, but milder than, those found for group B	

with presence of necrotic foci, and serous inflammation of the CAM. Even the surviving embryos were retarded in growth and part of them had discoloured yolk (Table II).

The pathological changes seen by light and electron microscopy are also summarized in Table II.

Signs indicative of virus replication (acidophilic intranuclear inclusion bodies, focal necroses or dystrophic areas with karyorrhexis and karyopycnosis) were seen in the liver, kidney, myocardium, gizzard muscle, spleen, and CAM of embryos infected with the virulent virus and those inoculated with the attenuated virus strain. In the third and fourth quarter of incubation an inflammatory cell reaction characterized by heterophilic granulocytes and lymphocytes plus histiocytes, respectively, joined the above-mentioned changes (Figs 4–9).

The majority of the above-listed changes were pronounced in embryos that had died of infection with the virulent virus. Contrarily, in embryos that died after inoculation with the attenuated virus strain these changes were less expressed. Only a certain part (40 and 50%, respectively) of the embryos

Table III

Results of cellular tests done at hatching with goose embryos* inoculated with duck plague virus strains of different virulence

Group of embryos (see Table I)	Lymphocyte stimulation**	Immunorosette formation***
C	10.7	2.2
B	39.5	22.0
D	11.8	7.3
Placebo-treated control (PC)	2.0	1.2
Untreated control	0.8	1.0

* mean value for 5 embryos each; ** number of cells showing blastogenic transformation/100 cells; *** immunorosette formation/ /100 μl cell suspension

surviving until hatching and killed at that time exhibited some of the above-mentioned lesions, and there were embryos which were completely free from pathomorphological changes.

Electron microscopy revealed herpesvirus particles intracellularly in the liver and myocardium of embryos killed on postinoculation (PI) day 5. The particles were seen in the nucleus of some cells showing characteristic changes of the chromatin substance (Fig. 10). In one embryo killed on PI day 10, virus particles were seen extracellularly in necrotic foci present in the gizzard wall and CAM, among the disintegrated cell components (Fig. 11).

The virus strains were consistently recovered from the organs of the dead embryos. The reisolation rate of viruses from surviving embryos killed at hatching was around 50%.

Specific VN antibodies were demonstrable neither in the yolk of the control embryos nor in the serum of the embryos surviving the infection and killed at hatching.

The results of *in vitro* cellular tests are shown in Table III. The lymphocytes separated from the blood of the infected embryos recognized the viral antigen in the immunorosette formation test and responded to the virus with blastogenic transformation.

Discussion

In goose embryos inoculated with DPV strains of different virulence pathomorphological changes of different severity but similar type developed. As a consequence of virus replication, these changes were seen in the liver, kidney, heart, gizzard and CAM. As opposed to adult birds (Jansen, 1968; Leibovitz, 1969; Friend and Pearson, 1973; Hofstad et al., 1978; Montgomery et al., 1980; Vetési et al., 1982), in the embryos the digestive tract mucosa

and the lymphoid organs did not suffer damage. This may be attributed to the fact that at the early stage of embryonic development the latter organs are rather poorly developed and little differentiated.

The severity of lesions depended on the virulence of the virus strain. Moreover, with both virus strains, the type of the lesions was influenced by the time of inoculation (day 12 or 20) and the time that elapsed between infection and death or killing. Thus, in embryos inoculated on day 12 of incubation and examined on day 15–19 after death or killing, signs indicative of circulatory disturbances, oedema and regressive changes due to virus replication were predominant. In embryos examined beyond day 20 (and inoculated either on day 12 or day 20 of incubation), signs of circulatory disturbances and oedema were less expressed, and the regressive changes were accompanied by cellular reactions characterized by heterophilic granulocytes, and from day 24–25 by those characterized by lymphocytes and histiocytes. A similar finding was obtained earlier in chicken embryos inoculated with lentogenic Newcastle disease virus and avian reovirus (Glávits et al., 1984). Namely, in the last quarter of embryonic life the colonization of lymphoid organs and tissues by lymphocytes commences both in chicken and in goose embryos (Glávits et al., 1981, 1987).

The mortality rate was influenced by the virulence of the virus strain used, the time of inoculation, and the virus dose. The majority of the surviving embryos had been inoculated with the less virulent strain, with a lower dose and at a later stage of embryonic development.

No specific VN antibodies were demonstrable in the serum of inoculated embryos that had survived up to hatching. However, the lymphocytes separated from their blood recognized the viral antigen *in vitro*, and responded to it with blastogenic transformation. This may be associated with the observation that antibodies to DPV appear rather late and persist for a short time even in adult birds. Such birds, however, were protected against infection even after the humoral antibodies had disappeared, indicating the possible involvement of cell-mediated immunity (Erickson et al., 1974; Dardiri, 1975).

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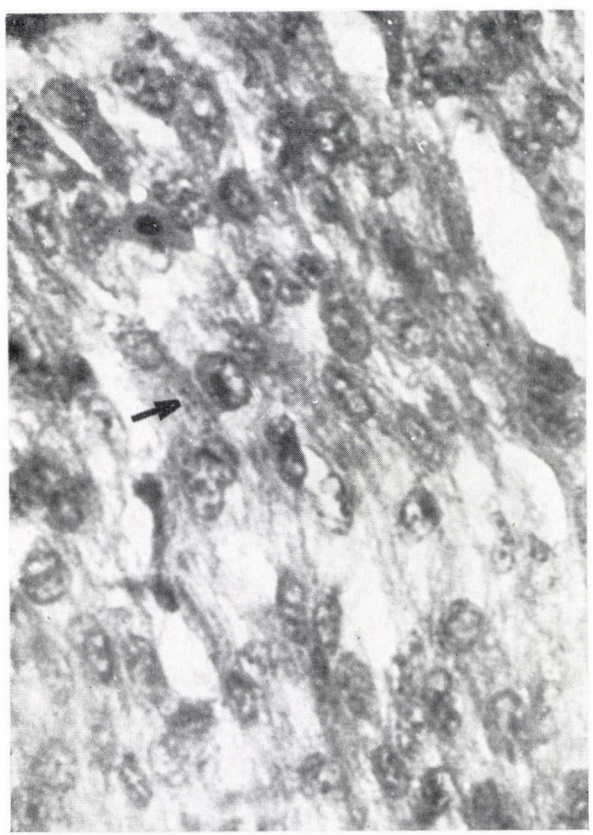
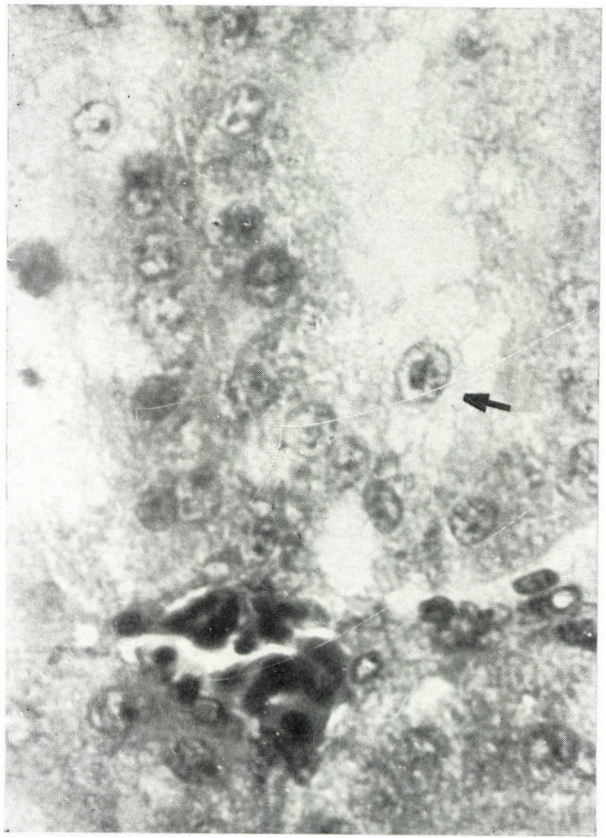
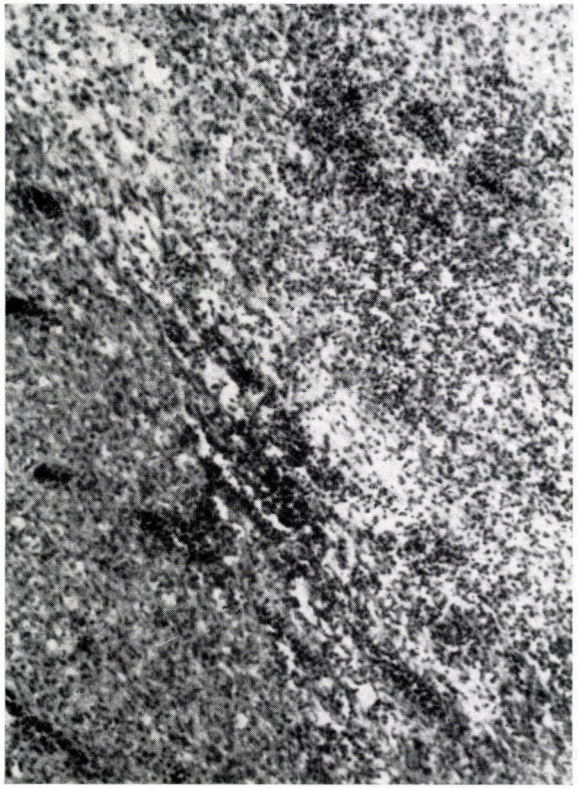
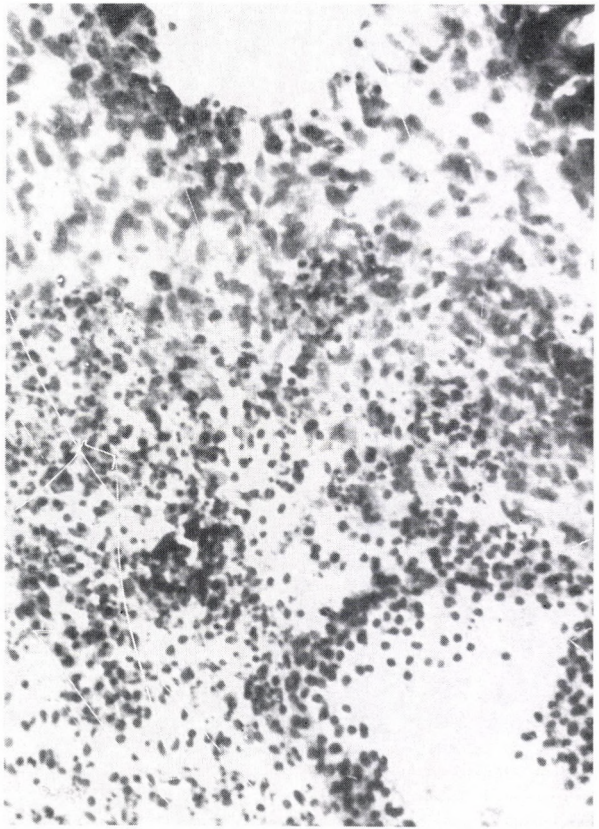
Fig. 3. Oedema and haemoglobinous infiltration in goose embryos inoculated with virulent duck plague virus on day 12 of incubation and killed 5 days later (on the left: control embryo)





Figs 4 and 5. Intranuclear inclusion bodies in a myocardial cell (↑) and in an epithelial cell of the renal tubules (↑) of goose embryos inoculated with virulent duck plague virus on day 12 of incubation and killed 5 days later. Haematoxylin and eosin, × 400





Figs 6 and 7. Gizzard muscle and CAM of goose embryos inoculated with virulent duck plague virus on day 12 of incubation and killed 10 days later. Note circumscribed necrosis with karyorrhexis and disintegration of the tissue structure. Haematoxylin and eosin, × 160 and × 63, respectively

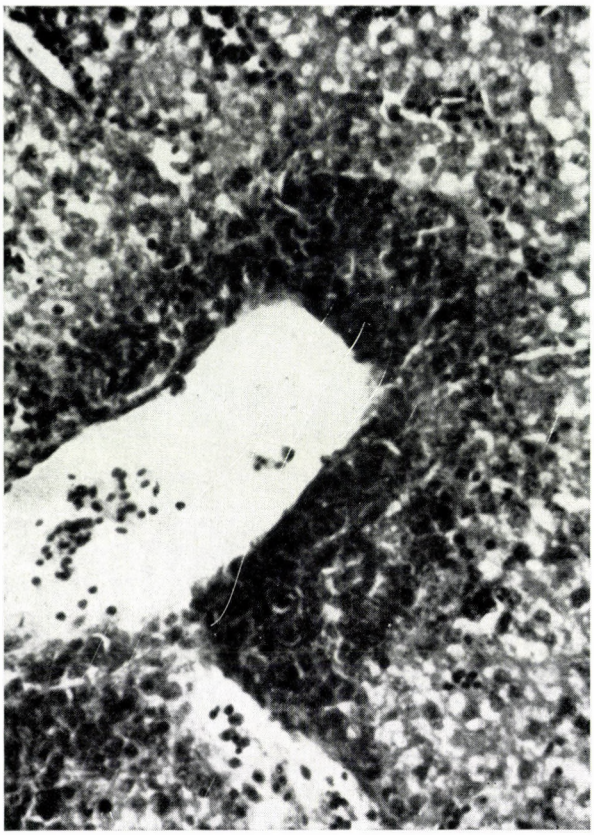


Fig. 8. Liver from a goose embryo inoculated with attenuated virus on day 12 of incubation and killed 10 days later. Note interstitial inflammation characterized by infiltration with heterophilic granulocytes. Haematoxylin and eosin, × 160

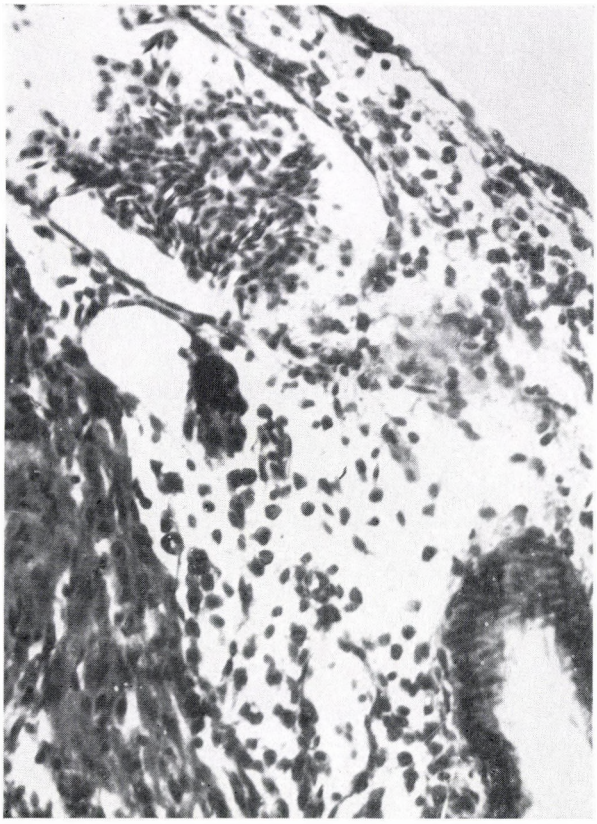


Fig. 9. Serous epicarditis in an unhatched embryo inoculated with virulent duck plague virus on day 20 of incubation. Haematoxylin and eosin, × 160

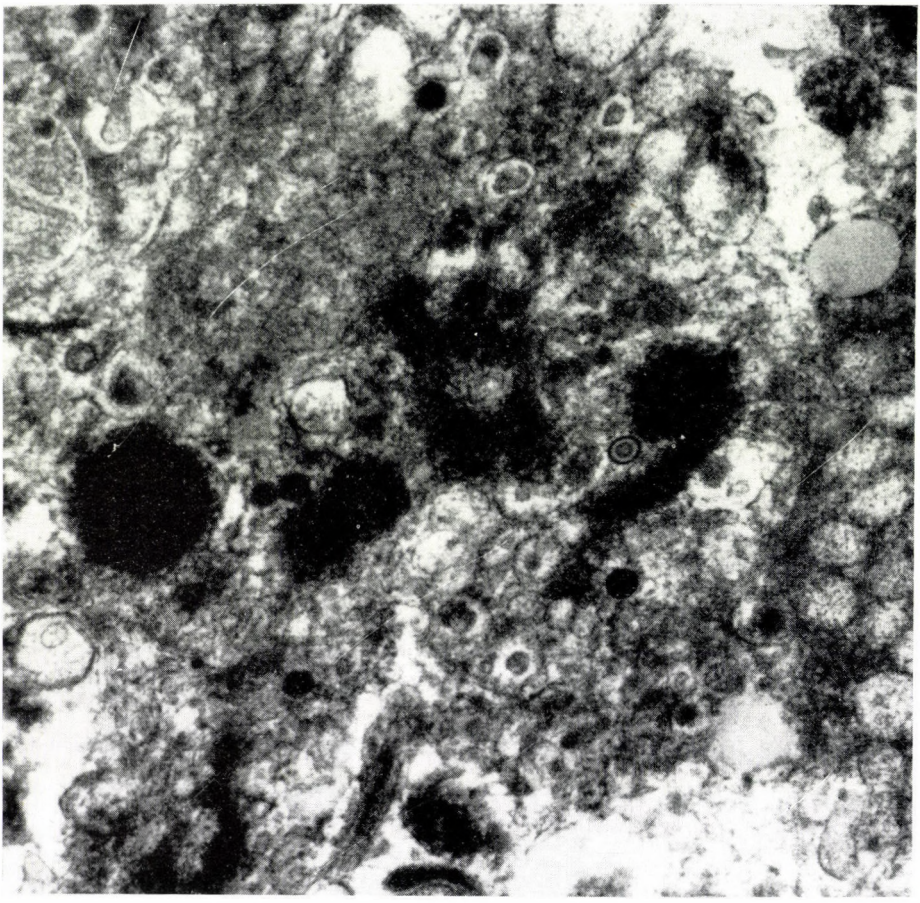


Fig. 10. Maturing herpesvirus particles in a hepatocyte from a goose embryo inoculated with virulent herpesvirus on day 12 of incubation and killed 5 days later. Electron micrograph, × 44,600

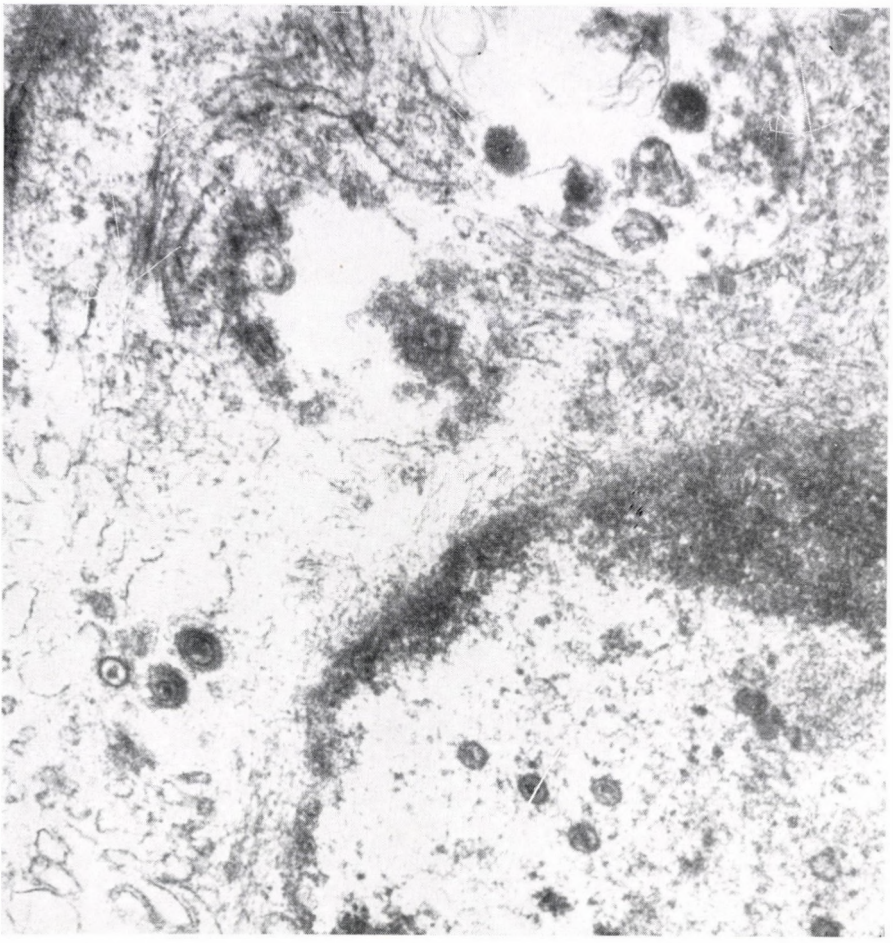


Fig. 11. Gizzard muscle from a goose embryo inoculated with virulent duck plague virus on day 12 of incubation and killed 10 days later. Note herpesvirus particles among the cell debris. Electron micrograph, × 44,600

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DIAGNOSTIC VALUE OF INTRAVENOUSLY ADMINISTERED JOHNIN PURIFIED PROTEIN DERIVATIVE (PPD) IN BOVINE PARATUBERCULOSIS

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The diagnostic value of intravenously administered johnin purified protein derivative (PPD) was studied in 45 cattle of different age, coming from herds infected by, or free from, *Mycobacterium paratuberculosis*.

In addition to observing the clinical symptoms, the animals' sera were assayed for specific antibodies by the complement fixation (CFT) and immunodiffusion (AGID) tests. The blastogenic transformation of peripheral lymphocytes was determined on the basis of ^{3}HTdR incorporation. Changes in the neutrophilic leucocyte/lymphocyte ratio of the blood were also monitored. Detection of the pathogen in the faeces was attempted by microscopic examination and by culturing.

Combined evaluation of responses elicited by intravenously administered johnin PPD can be a valuable aid in recognizing infected animals, particularly those among the heifer progeny of infected cows.

Keywords: Bovine, paratuberculosis, intravenous johnin PPD, diagnosis

Due to the unique pathogenesis and immunopathology of paratuberculosis, no diagnostic method is available which can reliably detect infection in all phases of the disease or by which infection could be ruled out with certainty.

The unreliability of immunodiagnostic tests results from the fact that while *Mycobacterium bovis* infection elicits the systemic form of cell-mediated immunity (Youmans, 1979; Chaparas, 1982; Hahn and Kaufman, 1981), *M. paratuberculosis* bacteria, multiplying intracellularly in the phagocytes of the intestinal mucosa, initially induce only local immune responses which may later be followed by systemic immune reactions depending on the progression of the disease.

This may be one of the reasons why the intradermal test done with paratuberculin fails to detect infection in the preallergic and preclinical phase of the disease. Another explanation can be that host cells do not recognize the antigen, or that antigen-induced clonal differentiation of the lymphocytes is delayed. It is also possible that, due to the constant reinfection, the bacterial

antigens cause a deficiency in the already sensitized cells (Ratledge and Stanford, 1983; Paliwal et al., 1985).

The unreliability of the immunodiagnostics of paratuberculosis is further increased by the possibility of cross-reactions attributable to the close antigenic relationship existing between *M. paratuberculosis* and *M. avium-intracellulare* strains (Tuboly, 1965; Körmendy et al., 1984; Saxegaard and Baess, 1988). This can appear, in addition to the diagnostic allergy tests, also as cross-reactivity of low-titre circulating antibodies detectable at the lepromatous stage of paratuberculosis (Duncan et al., 1978; Merkal, 1973; Thorel, 1981).

Searching for new diagnostic possibilities, the applicability of the lymphocyte stimulation test (LST Alhaji et al., 1974; Bergman, 1980), the diagnostic value of intravenously administered johnin (Larsen and Kopecky, 1965), and the haematological changes produced by intravenously administered johnin purified protein derivative (PPD) (Hall and Thoen, 1983; Benedictus and Bosma, 1985) were studied earlier.

In the present work, the organic and focal reactions elicited by intravenously administered paratuberculin, the accompanying haematological changes, the LST index, and results of serological and faecal examinations were studied in cattle of different age and kept under different epizoological conditions.

Material and methods

Animals

Forty-five cattle of different breed and age were used. Of them, 10 cows showed the clinical symptoms of paratuberculosis and were bacteriologically positive (Table I). Twenty-five cows came from an infected herd: of them, 15 and 10 cows were positive and negative, respectively, by the complement fixation test (CFT) 3 weeks before the experiment (Table II and Table III). Five 1-year-old heifers that had been born in an infected herd (Table IV) and five 1-year-old heifers that had come from an infection-free herd (Table V) were also used in the experiment. All experimental animals belonged to the Holstein-Friesian breed.

Clinical examinations

At the start of the experiment (0 hour) the clinical status of the animals (disease symptoms, body temperature, heart rate, respiratory rate, rumen motility) was recorded. Subsequently 3 ml paratuberculin (0.5 mg/ml tuberculoprotein PPD, Phylaxia Veterinary Biologicals Co., Budapest) was injected intravenously. Prior to the injection of paratuberculin, blood samples were taken from all animals into heparinized and anticoagulant-free tubes for the

baseline examinations. Following clinical examination performed in the 6th hour after the injection of johnin PPD, blood and faecal samples were taken from the animals.

Haematological examinations

The differential blood count was determined as described by Ormay (1961).

Lymphocyte stimulation test (LST)

Lymphocytes were separated with Ficoll-Paque from blood samples taken into heparinized tubes, suspended in Hanks' solution containing 10% fetal calf serum, and cell density was adjusted to 10^6 per ml. The suspensions were measured into Leighton tubes, incubated at 37 °C for 72 h, and in the 56th h the cell cultures were supplemented with 10 μCi ^{3}HTdR. The rate of blastogenesis was evaluated by autoradiography (Tuboly, 1979).

Serological tests

The complement fixation test (CFT) and double radial immunodiffusion (AGID) test were carried out as described by Körmendy et al. (1984). The antigen and reference serum used in the CFT were produced and marketed by Phylaxia Veterinary Biologicals Co., Budapest.

Microscopic examination of the faeces

Microscopic examination of the faeces was performed according to Hungarian Standard MSZ 419-83. One g of faeces was suspended in 4 ml of saline and shaken with 2 ml of chloroform. Three drops from the chloroform phase were placed on slide, dried and stained with Ziehl-Neelsen stain.

Bacteriological examinations

Herold's medium containing mycobactin was used. The samples were decontaminated according to Beerwerth (1967). The inoculated media were incubated at 37 °C for 150 days. The isolates were identified according to Thorel and Valette (1976).

Results

The results obtained for clinically ill cattle coming from an infected herd are shown in Table I. At 6 h after the injection of paratuberculin, organic and focal reactions (muscle tremors, severe diarrhoea, elevated heart rate and respiratory rate) were present but body temperature was practically unaltered

(mean elevation: 0.07 °C). The neutrophilic leucocyte/lymphocyte ratio underwent a 5- to 10-fold (in average: 9-fold) increase in all animals except cow no. 2. The blastogenic transformation of lymphocytes increased by 3 to 6% as compared to the baseline value. In the 6th hour no changes from the baseline values were demonstrable in humoral antibody levels by AGID and CFT. As a result of the injection of paratuberculin the number of cattle excreting *M. paratuberculosis* bacteria in quantities demonstrable in the faeces by microscopy rose from 3 to 6. Bacteriological examination of the faeces proved the presence of *M. paratuberculosis* in 8 animals.

Table I

Organic reaction of cows showing

Number and status of animal	Serial number	0 hour							
		Clinical symptoms	Heart rate	Respiratory rate	Body temperature °C	Rumen motility	LST index, in %	Ratio of neutrophils + lymphocytes	AGID
3071 5 years old, at 4 months of gest.	1	—	72	—	38.8	9	17	0.42	+ day 2
3169 8 years old, at 3 months of gest.	2	—	63	23	38.0	13	7	1.10	—
3120 5 years old, non-pregnant	3	pharyng. oedema	54	42	38.5	6	8	0.31	—
3340 4 years old, at 3 months of gest.	4	pharyng. oedema	78	35	38.8	4	12	0.67	+
3583 3 years old, at 4 months of gest.	5	—	48	54	38.9	10	10	0.63	+
3196 4 years, at 5 months of gest.	6	+ diarrh.	84	40	39.0	9	11	0.25	—
3478 4 years old, at 4 months of gest.	7	—	78	—	39.0	7	15	0.32	+ day 2
3487 4 years old, non-pregnant	8	—	92	48	38.9	7	3	0.34	—
3574 3 years old, at 4 months of gest.	9	—	94	54	38.9	3 weak	8	0.53	—
3182 4 years old, at 7 months of gest.	10	—	76	51	38.7	10	6	0.41	—

CFT = complement fixation test; * serum dilutions in the CFT; AGID = agar gel immunodiffusion; gest. = gestation; saliv. = salivation; pharyng. = pharyngeal; diarrh. = = diarrhoea

The results obtained for clinically negative animals positive by blood test ad/or faecal examination are shown in Table II. In that group mild organic reactions were observed. The faecal samples of 7 and 11 out of the 15 cattle contained acid-fast bacteria in nest-like arrangement at 0 and 6 h, respectively. The animals' body temperature did not deviate from the initial (baseline) values. On the other hand, the neurophil/lymphocyte ratio increased in all but one of the 12 animals (average increase: 0.505). The degree of this increase varied by animal. The serum level of complement fixing antibodies and antibodies demonstrable by AGID did not change.

the clinical symptoms of paratuberculosis

6 h												
CFT 1 : 5, 1 : 10, 1 : 20*	Microscopic examination of the faeces	Clinical symptoms	Heart rate	Respiratory rate	Body temperature °C	Rumen motility	LST index, in %	Ratio of neutrophils + lymphocytes	AGID	CFT 1 : 5, 1 : 10, 1 : 20*	Microscopic examination of the faeces	Bacteriological examination of the faeces
4 4 3	+	+ saliv. muscle tremors	78	45	38.8	12	22	11.0	+ day 2	444	+	+
—	—	+	104	42	38.7	2	12	1.52	—	—	—	—
4 4 1	—	—	102	60	38.8	10	14	4.01	—	2	+	+
4 3 1	+	diarrh. muscle tremors	78	48	39.0	4 weak	17	1.37	—	441	+	+
4 3 1	—	diarrh. muscle tremors	112	62	39.0	7	8 plasma	3.92	+	442	—	+
4 3 1	—	severe diarrh. muscle tremors	84	60	39.0	6	14	4.36	—	431	—	+
4 3 2	+	saliv.	90	56	38.8	4	19	12.50	+ day 2	431	+	+
—	—		108	48	38.7	7 weak	9	1.19	—	—	—	—
—	—		69	58	38.6	10	14	2.00	—	—	+	+
—	—		88	56	38.8	11	12	3.35	—	—	+	+

Table II

Organic reaction of cows positive for paratuberculosis

Serial number	No. of animal	Age (years)	0 h									
			Heart rate	Respiratory rate	Body temperature °C	Rumen motility	Ratio of neutrophils + lymphocytes	AGID	CFT			
									1 : 5	1 : 10	1 : 20	1 : 40
11	1500	8	70	18	38.6	6	0.31	—	4	2	—	—
12	2193	5	74	17	38.6	9	0.38	—	4	4	2	—
13	2188	5	82	24	39.0	11	0.34	—	3	1	—	—
14	1664	6	73	29	38.7	8	0.51	+	4	4	2	—
15	2335	5	67	20	38.8	7	0.40	—	4	4	4	2
16	2053	6	84	26	38.2	13	0.48	—	4	4	3	—
17	1922	6	80	23	38.7	8	0.33	—	4	2	—	—
18	2020	5	76	22	38.5	7	0.40	—	4	4	2	—
19	1097	9	84	28	38.9	7	0.34	—	4	4	4	3
20	1149	9	75	24	38.4	12	0.25	—	4	4	4	2
21	2216	5	72	20	38.4	6	0.55	—	4	4	2	—
22	1853	6	81	19	38.1	9	0.35	—	4	4	1	—
23	2723	4	80	31	38.6	10	0.15	—	—	—	—	—
24	2154	5	79	19	38.3	11	0.31	—	4	1	—	—
25	2152	5	78	22	38.4	8	0.48	—	—	—	—	—

Table III

Organic reaction of cows negative for paratuberculosis

Serial number	No. of animal	Age (years)	0 h									
			Heart rate	Respiratory rate	Body temperature, °C	Rumen motility	LST %	Ratio of neutrophils + lymphocytes	AGID	CFT		
										1 : 5	1 : 10	1 : 20
26	2484	6	72	22	38.4	11	6	0.76	—	—	—	—
27	3051	5	76	19	38.4	8	—	0.66	—	—	—	—
28	3029	5	68	21	38.3	12	—	1.30	—	4	1	—
29	2225	7	67	22	37.1	7	—	0.29	—	—	—	—
30	3584	3	80	17	38.5	9	—	0.34	—	—	—	—
31	3007	5	82	28	38.3	14	—	0.77	—	—	—	—
32	3511	4	79	32	38.2	6	7	0.45	—	—	—	—
33	2025	8	84	26	37.9	—	—	1.06	—	—	—	—
34	3708	3	75	18	38.1	10	—	0.62	—	—	—	—
35	3429	4	74	21	37.2	7	—	0.74	—	—	—	—

by blood test or faecal examination

6 h											
Microscopic examination of the faeces	Heart rate	Respiratory rate	Body temperature °C	Rumen motility	Ratio of neutrophils + lymphocytes	AGID	CFT 1 : 5	CFT 1 : 10	CFT 1 : 20	CFT 1 : 40	Microscopic examination of the faeces
+	74	21	39.0	6	1.59	—	4	3	—	—	+
+	76	24	38.6	8	1.11	—	3	3	1	—	+
—	76	21	38.8	12	1.75	—	3	1	—	—	—
+	75	20	38.2	9	1.11	+	4	4	—	—	+
—	80	19	38.9	9	0.19	—	4	4	4	3	—
+	82	17	38.3	10	1.13	—	4	4	3	—	+
—	76	23	39.0	11	0.45	—	4	3	1	—	—
—	77	29	38.3	10	1.14	—	4	4	2	1	+
—	81	21	38.6	14	0.16	—	4	4	4	3	+
—	69	24	38.4	8	0.56	—	4	4	3	—	+
—	68	26	38.7	7	0.56	—	4	3	1	—	—
+	73	23	38.1	9	1.09	—	4	3	1	—	+
+	80	25	38.6	11	0.35	—	—	—	—	—	+
—	74	27	38.6	9	1.26	—	4	2	—	—	+
+	70	20	38.5	8	0.71	—	1	—	—	—	+

by serological tests and faecal examination

6 h											
Microscopic examination of the faeces	Heart rate	Respiratory rate	Body temperature, °C	Rumen motility	LST %	Ratio of neutrophils + lymphocytes	AGID	CFT 1 : 5	CFT 1 : 10	CFT 1 : 20	Microscopic examination of the faeces
—	76	26	38.4	8	5	1.00	—	—	—	—	—
—	75	18	37.9	10	—	1.20	—	—	—	—	—
—	80	22	38.6	10	—	1.54	—	4	1	—	—
—	81	24	38.4	8	6	0.48	—	—	—	—	+
—	83	24	38.3	11	9	1.30	—	—	—	—	—
—	69	28	38.2	10	6	1.00	—	—	—	—	—
—	77	34	38.0	10	—	0.32	—	—	—	—	—
—	74	30	37.8	9	—	2.06	—	—	—	—	—
—	79	24	38.5	8	—	1.00	—	—	—	—	—
—	80	36	30.3	11	—	0.79	—	—	—	—	—

Animals of group 3 (Table III) did not show signs of organic reaction on clinical examination. However, *M. paratuberculosis* bacteria were demonstrable in the faeces of 2 animals (no. 29 and 35). The body temperature of these animals rose by 1.3 and 1.1 °C, respectively. Cow no. 29 had an LST index of 6%. The average rise of body temperature for the group was 0.2 °C. The neutrophilic leucocyte/lymphocyte ratio rose by 0.37 on the average: this increase varied by individual. In the 6th hour, the rate of blastogenic transformation determined by LST was 9% for cow no. 30 and 5 to 6% for samples from cows nos 26, 29 and 31. The baseline value measured in the sample of cow no. 32 (7%) decreased to 0 by the 6th hour after the injection of paratuberculin.

Table IV

Organic reaction of heifers born in a cow herd infected

Serial number	No. of animal	Age (years)	0 h									
			Heart rate	Respiratory rate	Body temperature, °C	Rumen motility	LST %	Ratio of neutrophils + lymphocytes	AGID	CFT		
										1 : 5	1 : 10	1 : 20
36	2992/7	1	68	34	38.7	8	—	0.09	—	—	—	—
37	2769/7	1	64	38	38.9	14	8	0.25	—	—	—	—
38	3470/7	1	72	46	38.9	12	6	0.44	—	—	—	—
39	3056/7	1	86	56	38.9	9	—	0.08	—	—	—	—
40	3518/7	1	80	50	38.8	7	NT	0.16	—	—	—	—

NT = not tested

Table V

Organic reaction of heifers born in a herd

Serial number	No. of animal	Age (years)	0 h									
			Heart rate	Respiratory rate	Body temperature, °C	Rumen motility	LST %	Ratio of neutrophils + lymphocytes	AGID	CFT		
										1 : 5	1 : 10	1 : 20
41	3569	3	73	20	38.3	11	—	0.54	—	—	—	—
42	3750	3	86	19	39.0	14	—	0.58	—	—	—	—
43	3767	3	80	28	38.6	9	—	0.79	—	—	—	—
44	3069	5	83	18	38.1	12	—	0.66	—	—	—	—
45	3711	3	79	23	38.4	7	—	1.01	—	—	—	—

In group 4 (Table IV) no organic reaction was observed, and the results obtained at 0 and at 6 h did not differ. However, the body temperature rose by 0.3 °C and the neutrophil/lymphocyte ratio increased by 0.080 (40%) on the average (from 0.204 to 0.284). Blood samples from 2 cows (no. 37 and 38) of this group showed a baseline LST index of 8 and 6%, respectively. By the 6th h these values rose to 18 and 10%, respectively.

Cows of group 5 (negative control) showed no organic reaction (Table V). The LST was negative but the leucocyte/lymphocyte ratio rose by 10% on the average (from 0.716 to 0.796).

by *Mycobacterium paratuberculosis*

6 h											
Microscopic examination of the faeces	Heart rate	Respiratory rate	Body temperature, °C	Rumen motility	LST %	Ratio of neutrophils + lymphocytes	AGID	CFT 1 : 5	CFT 1 : 10	CFT 1 : 20	Microscopic examination of the faeces
+	62	48	38.8	9	—	0.23	—	—	—	—	—
+	88	40	39.1	6	18	0.35	—	—	—	—	—
—	86	42	39.3	8	10	0.09	—	—	—	—	—
+	96	40	39.6	14	—	0.43	—	—	—	—	—
—	78	42	38.7	7	—	0.32	—	—	—	—	—

negative for paratuberculosis

6 h											
Microscopic examination of the faeces	Heart rate	Respiratory rate	Body temperature, °C	Rumen motility	LST %	Ratio of neutrophils + lymphocytes	AGID	CFT 1 : 5	CFT 1 : 10	CFT 1 : 20	Microscopic examination of the faeces
—	74	24	38.6	8	—	0.84	—	—	—	—	—
—	80	20	38.4	9	—	0.99	—	—	—	—	—
—	72	42	38.5	12	—	0.34	—	—	—	—	—
—	68	23	38.1	13	—	0.61	—	—	—	—	—
—	81	21	38.1	10	—	1.20	—	—	—	—	—

Discussion

Intravenously administered paratuberculin induced organic reaction and haematological changes of varying degree in cattle of different epizootiological and clinical status. Clinically ill animals (Table I) and animals positive by blood test (Table II) showed not only organic reaction but also an increase in the rate of blastogenesis by LST and elevated neutrophil counts. In the group of clinically ill cows the allergen induced a 9-fold increase in the neutrophil/lymphocyte ratio. In the serologically positive group this ratio increased by 0.5, in the group originating from an infected herd but serologically negative (Table III) it increased by 0.37, while in heifers suspected of being infected (Table IV) it rose by 0.08, to a similar degree as in the negative heifers.

The LST was positive in the clinically ill animals and the LST index increased. This test was not done with the serologically positive group, for the epizootiological evaluation of that group does not pose a problem.

In evaluating the heifer progeny of the infected herd it seems to be important that the LST identified 2 animals (nos 37 and 38) as being infected already at the baseline testing. This result was confirmed by the examination of faecal samples taken in the 6th h, proving that the suggestion of Amstutz (1984) and Chiodini et al. (1984) is right: namely, that the heifer progeny of infected cows should be reared separately and can be bred only after the favourable result of several screening tests if paratuberculosis is to be eradicated from the herd.

It should be noted, however, that none of the applied methods is absolutely reliable in demonstrating infection-free status, as e.g. the neutrophil/lymphocyte ratio increased to a similar degree in the negative animals and in the infected heifers.

As a result of the organic reaction, the detectability of *M. paratuberculosis* in the faeces increased.

Paratuberculosis did not induce changes in the antibody levels demonstrable by CFT and AGID (animals nos 3 and 4 were an exception).

The average rise in body temperature induced by the intravenously administered antigen was 0.1, 0.2 and 0.3 °C in the clinically ill group, in the serologically negative animals living in infected environment (Table III), and in heifers from an infected herd (Table IV), respectively. The average body temperature of serologically positive cows (Table II) and negative animals (Table III) remained practically unchanged. These observations are in contradiction to data obtained by Benedictus and Bosma (1985), who found a 1.4 °C rise in the body temperature of 84% of the clinically ill animals 6 hours after injection of paratuberculin.

On the basis of the present studies it appears that simultaneously applied diagnostic procedures can make assessment of the infection status of a given herd for paratuberculosis more accurate and reliable.

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EVALUATION OF CELL-MEDIATED IMMUNITY DURING CHRONIC ORGANOPHOSPHATE PESTICIDE INTOXICATION IN MICE AND GOATS

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The status of cell-mediated immunity (CMI) after pesticide exposure was assessed in mice with the help of skin sensitivity and graft versus host reaction tests. It was observed that at 24 hours post-challenge CMI values did not differ significantly from the control, indicating no effect of quinalphos treatment in mice. Goats receiving monocrotophos at a dose rate of 1.0 mg kg^{-1} body mass for 40 days gave a similar result when CMI was tested with the help of the chemical sensitizer dinitrofluorobenzene (DNFB). Thus the results clearly indicate that the tested organophosphates do not interfere with cellular immunity in the intoxicated animals.

Keywords: Cell-mediated immunity, chronic organophosphate intoxication, pesticide, mouse, goat

Contradictory reports are available on the immune response of animals treated with different organophosphate pesticides. Dimov and Simeonov (1985) observed a stimulatory effect on the immune system of sheep after exposure to phosalone. On the other hand, Casale et al. (1984) reported suppression of humoral immunity in inbred mice treated with parathion. No information is available about the effect of quinalphos and monocrotophos on cell-mediated immunity (CMI). The present communication reports the status of CMI in mice and goats during chronic quinalphos and monocrotophos intoxication, respectively.

Materials and methods

Mouse experiment

A total of 26 male Swiss albino mice were used for this experiment. They were kept on a normal balanced diet and caged singly. After one month of observation the mice were divided into two groups.

Group I (control) consisted of 12 mice which received intraperitoneal (i.p.) injection of sterilized normal saline solution (NSS) for 30 days. They acted as control for the treated group of mice.

Group II (quinalphos-treated) had 14 mice which received intraperitoneal injection of quinalphos at a dose rate of 3.75 mg kg^{-1} body mass every day for 30 days.

Assessment of CMI

Contact chemical sensitization of the skin is regarded as a form of CMI (Gell and Benacerraf, 1961). 2,4-dinitrofluorobenzene (DNFB) was used for contact sensitization by the method described by Phanuphak et al. (1974). The mice were sensitized with DNFB by a single intracutaneous application of 0.1 ml (10 mg ml^{-1}) in acetone vehicle. An area of 0.25 cm^2 on the dorsal aspect of both ears was marked for the application. DNFB was applied on the marked area of the right ear drop by drop and allowed to evaporate. The left ear served as control onto which only vehicle was applied. Primary sensitization was done on the 8th day of intoxication.

After 16 days of primary sensitization the sensitized mice were challenged with 0.1 ml of DNFB (1 mg ml^{-1}) on the sensitized site while the left ear was treated with vehicle only.

Ear thickness at the previously marked area was measured with the help of a slide-gauge at 0, 24 and 48 h post challenge with three readings each. The increase in mean ear thickness was obtained by deducting the ear thickness of the same site before challenge. The overall mean ear thickness (MET) was obtained by taking the mean of individual mice within the group.

Graft versus host reaction (GVHR). A total of 14 male mice aged about 1 month were used for this study. They were divided into two groups. Group I (control) comprised 6 mice which received an intraperitoneal injection of sterilized NSS. Group II (treated) consisted of 8 mice which were injected i.p. with quinalphos at a dose rate of 3.75 mg kg^{-1} body mass daily for 30 days. Subsequently, the mice were killed and their spleens were taken out in 0.5 ml of Hanks solution.

Twenty day-old chicks of the same breed and hatch were taken and divided into 3 groups, viz. negative control, control, and intoxicated group. Chicks of the negative control group received no treatment, whereas the control and intoxicated groups of chicks received 0.2 ml of splenic suspension from group I and II mice respectively, according to the method of Hudson and Hay (1980). All experimental chicks were killed after 96 h and the spleen of each chick was weighed carefully.

The splenic index of all the chicks was calculated by dividing spleen mass with body mass and multiplying the value with 100. The reaction of splenic cells of chicks in response to the splenic T cells of mice ultimately caused an increase in the size and mass of chicken spleen.

Goat experiment

A total of 10 male, uncastrated, clinically healthy Black Bengal goats aged about 6 months and weighing 6–10 kg were used for this experiment. After one month of observation they were divided into two unequal groups. Group I consisted of 3 animals which acted as control for group II animals and were drenched with distilled water at a dose rate of 0.5 ml kg^{-1} body mass. In group II there were 7 animals which were drenched with a distilled water suspension of monocrotophos at a dose rate of 1.0 mg kg^{-1} body mass for 40 days.

Assessment of CMI

This was carried out by contact sensitization on the skin of goats by 2,4-dinitrofluorobenzene as described by Phanuphak et al. (1974) in mice, later modified by Singh (1986) for goats.

A relatively hairless area of 20 cm^2 skin surface was shaved and cleaned on the left and right side of the neck. The goats were sensitized on the right side with DNFB (supplied by Sigma Chemical Co., U.S.A.) by a single intracutaneous application of 0.25 ml of DNFB (10 mg ml^{-1}) in a vehicle consisting of a 4 : 1 mixture of acetone and olive oil. The shaved area on the left side served as control for the experiment. Primary sensitization was done on the 22nd day of intoxication.

After 15 days of primary sensitization (i.e. on the 37th day of intoxication) the sensitized goats were challenged with 0.25 ml of DNFB (0.1 mg ml^{-1}) onto the sensitized area, while the left side received vehicle only.

Thickness of the skin was measured at both sites with the help of vernier callipers at 0 h (i.e. prior to challenge) and at 6, 12, 24 and 48 h post challenge. Three readings from each goat were obtained at every hour of measurement. Mean skin thickness (MST) was calculated from these readings and Student's *t* test was applied to test the difference, if any, in CMT between the two groups of goats at 6, 12, 24 and 48 h after challenge, according to the formulae recommended by Snedecor and Cochran (1968). The weighed *t* test was also applied in the case of non-homogeneity of variance.

Results

DNFB test

Mice. The mean increase in ear thickness at different times post challenge for both groups of mice is shown in Table I.

At 6, 24 and 48 hours the thickness of the challenge site (right ear) differed significantly from that of the control site (left ear). However, at no

Table I

Mean increase in ear thickness (mm) and "t" values at different times after challenge of mice with DNFB

Hours after challenge	Site	Groups		"t" values		
		Normal control (Group I)	Quinalphos-treated (Group II)	Left × Right (Group I)	Left × Right (Group II)	Left × Right (Group I and II)
6 hours	Left control)	0.0455±0.0149	0.019±0.0072			
	Right (challenged)	0.1336±0.038 (11)	0.176 ±0.0257 10)	2.159*	5.88*	0.91^{NS}
24 hours	Left (control)	0.0227±0.015	0.0466±0.0197			
	Right (challenged)	0.2091±0.035 (11)	0.1511±0.0288 (9)	4.90**	3.47**	1.52^{NS}
48 hours	Left (control)	0.032±0.0133	0.022±0.0081			
	Right (challenged)	0.136−0.0331 (10)	0.1311±0.0238 (9)	2.97**	4.364**	0.12^{NS}

^{NS} = non-significant; * = significant ($P < 0.05$); ** = highly significant ($P < 0.01$)

stage of the experiment did the treated mice differ from the control ones when the challenged sites of the two groups were compared statistically. This clearly pointed out that quinalphos treatment of 4 weeks duration did not alter the cell-mediated immune potentials of mice.

Goats. The increase in skin thickness reached its peak at 24 h after application of the challenge dose of DNFB and the challenged site differed significantly from the control site. At no stage of the experiment did the

Table II

Mean increase in skin thickness (mm) and "t" values at different times after challenge of goats with DNFB

Hours	Site	Groups		"t" value		
		Normal control (Group I)	Monocrotophos--treated (Group II)	Left vs. Right (Group I)	Left vs. Right (Group II)	Right vs. Left Group I and II)
6 hours	Left (control)	0.011±0.0109	0.0046±0.0151			
	Right (challenged)	0.133±0.0507 (3)	0.4476±0.1139 (7)	2.35^{NS}	3.85^{NS}	1.75^{NS}
12 hours	Left (control)	0.00033±0.0382	0.0187±0.0297			
	Right (challenged)	0.955±0.4223 (3)	0.819±0.1386 (7)	2.25^{NS}	5.64*	1.22^{NS}
24 hours	Left (control)	0.00033±0.0191	0.0047±0.0311			
	Right (challenged)	1.611±0.2983 (3)	1.2286±0.1298 (7)	5.386*	9.167*	1.41^{NS}
48 hours	Left (control)	0.00033±0.0191	0.043±0.0259			
	Right (challenged)	0.511±0.1746 (3)	0.6526±0.1621 (7)	2.90^{NS}	3.812^{NS}	0.512^{NS}

^{NS} = non-significant; * = significant ($P < 0.05$)
Figures in parentheses indicate the number of animals

Table III

Mean values of splenic index (SI) of different groups of chicks during the GVHR test

	Negative control	Control	Intoxicated	Negative control vs. control	Negative control vs. intoxicated	Control vs. intoxicated
Body mass of chicks (g)	49.583 ±1.619 (6)	48.50 ±1.088 (6)	46.750 ±1.218 (8)			
Spleen mass of chicks (g)	0.024 ±0.001 (6)	0.033 ±0.005 (6)	0.036 ±0.005 (8)			
Splenic index of chicks	0.049 ±0.004 (6)	0.069 ±0.011 (6)	0.077 ±0.011 (8)	1.709	2.393*+	0.503

NS = non-significant; * = significant at $P < 0.05$; + = Weighted "*t*" test

monocrotophos-treated animals differ from the control goats in skin thickness of the challenged sites (Table II). This indicated that regular drenching of monocrotophos to goats does not have any influence on the CMI.

GVHR test

The mean values of splenic index of chicks obtained in the GVHR test are shown in Table III. The splenic index of chicks inoculated with splenic suspension from control and intoxicated mice was apparently higher than that of negative control chicks. This clearly indicated that i.p. inoculation of splenic T cells from mice caused a definite reaction in the chick spleen. The elevation of splenic index in chicks receiving a splenic suspension from quinalphos-intoxicated mice was statistically significant ($P < 0.05$). On the contrary, the splenic indices of chicks receiving splenic suspension from intoxicated mice did not differ significantly from those of chicks receiving T cells from control mice. Thus no difference could be recorded between the CMI of control and quinalphos-treated mice.

Discussion

It was reported by Dimov and Simeonov (1985) that fenitrothion and phosalone dosing to sheep enhanced the agglutinin titres and phagocytic activity of the cells. Katsenovich et al. (1981) observed that human patients exposed to pesticides showed alterations in the ratio of T and B lymphocyte populations. There was a quantitative deficiency in T lymphocytes accompanied by a reduction in their functional activity. On the contrary, the number of B lymphocytes was increased. On the basis of an *in vivo* study, Tamang et al. (1988) reported immunosuppression in mice and goats due to cyper-

methrin intoxication. Immunodeficiency was demonstrated in experimental calves fed with bracken fern (Rao et al., 1987). Casale et al. (1984) reported suppression of humoral immunity in inbred mice after exposure to parathion (an organophosphate pesticide). Thus, it was essential to study the immunogenic status of organophosphate-treated animals with a view to settle the controversy.

The results of skin sensitivity test in mice and those of GVHR test in chicks treated with splenic T cells from mice are in agreement with each other. Although a small difference was recorded between the control and quinalphos-treated groups but statistically significant differences were found neither in the DNFB skin sensitivity test nor in the graft versus host reaction test. The DNFB skin sensitivity test in goats also revealed that CMI was not affected by monocrotophos intoxication. It is possible that some factors other than pesticide treatment might have affected the CMI in previous studies by other workers. It might also be true that individual members of the organophosphate group of pesticides differ in their immunogenic effect or that prolonged chronicity of experiments is essential to alter the immunological potency of animals. However, it is safe to conclude that quinalphos treatment of 4 weeks duration in mice and monocrotophos drenching of 40 days duration in goats did not cause any significant change in the CMI values.

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MYCOTOXIN RESEARCH IN THE HUNGARIAN CENTRAL VETERINARY INSTITUTE

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Mycotoxin research conducted in the Hungarian Central Veterinary Institute is reviewed briefly. The effect of zearalenone was studied by experiments in several animal species (cattle, pig, sheep, chicken, goose, duck, guinea fowl, fish) and in cell cultures *in vitro*. The chicken *(Gallus domesticus)* has proved to be resistant to the toxin. In the susceptible species zearalenone causes the most severe damage to the sexual organs.

The metabolism of zearalenone, T-2 toxin and diacetoxyscirpenol was also studied. These toxins are resistant to physicochemical factors but are easily transformed in biologically active environment.

The acute toxicity study of trichothecene toxins is described along with pathomorphological changes, with particular respect to those of the lymphoid and myeloid organs. Trichothecene toxins impair the natural defence mechanism of the organism and result in the manifestation of different diseases (swine dysentery, caecal coccidiosis).

Deoxynivalenol contamination of the feeds, first of all of wheat, is common in Hungary. Its effect on pig fattening was investigated in a field trial.

The toxins of different storehouse moulds were also examined.

Keywords: Mycotoxin research, zearalenone, T-2 toxin, deoxynivalenol, trichothecene toxins

Research and routine work on *aflatoxins* was started by us approximately 20 years ago in connection with mass mortality among ducklings fed an imported feedstuff. Then we tested the aflatoxin-producing ability of numerous mould strains and established that under our climatic conditions the formation of aflatoxin is not probable (Nyiredy and Bodnár, 1966). Nowadays only imported feedstuffs (e.g. ground nut meal and soya) are analysed for aflatoxin. In this field we do not perform any research work.

At the end of the sixties another problem arose. At big state farms the progeny was not satisfactory and this caused severe economic losses. Mycological examinations elucidated that the feedstuffs were contaminated with fusaria, and by chemical analysis a hormone-like substance, *zearalenone*, was detected. Extensive research began with feeding trials in various animal species (Ványi et al., 1973). Pathological and histopathological examinations revealed that zearalenone severely impaired ovarian and uterine function (Fig. 1) in cows and pigs and caused reproductive disturbances or infertility (Ványi et al., 1974*a*, *b*). In experiments with ganders a reduction in the number

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and quality of spermatozoa was found. The same was observed in carp, rams (Fig. 2), bulls and boars (Fig. 3) fed with corn containing zearalenone (Ványi et al., 1974*c*; Ványi et al., 1980; Ványi and Széky, 1980). Only the chicken showed resistance.

These observations demanded elaboration of a sensitive laboratory method for testing feed samples for zearalenone.

The cytotoxic effect of zearalenone in various monolayer cell cultures was suitable for this purpose (Ványi and Romváry-Szailer, 1974). In animals in which spermatogenesis is hindered by zearalenone, the testicular tissue is sensitive to the toxin. By this method the Rhesus monkey also showed sensitivity (Fig. 4), suggesting that men may also be sensitive to zearalenone.

We studied the metabolism of zearalenone in pregnant sows. The conclusions were as follows:

(i) The toxin given orally with the feed is completely absorbed and transported to the liver where it is reduced to zearalenol and becomes bound as glucuronate.

(ii) The bulk of the toxin is eliminated from the liver into the bile, then it gets back into the intestine and is excreted with the faeces.

(iii) A small part is reabsorbed from the intestine, gets into the circulation and is excreted with the urine.

(iv) In lactating animals a negligible quantity is excreted into the milk (Ványi et al., 1981–1983).

In large-scale pig herds it was observed that if the sows had ingested zearalenone during gestation, their piglets were born with swelling of the vulva. This is the so-called perinatal oestrogen syndrome. The sows form a depot from zearalenone, this is mobilized at farrowing and is excreted with the colostrum. The other part of the toxin is deposited in the fetus and can be detected from the liver of neonatal piglets.

We have conducted experiments with other *Fusarium* metabolites, too. We investigated the effect of *T-2 toxin* on broiler chickens between the age of two and eight weeks. Chickens fed a feedstuff containing 0.6 ppm T-2 toxin showed feathering disturbances and ulcer formation in the mouth and on the tongue. The histopathological examinations showed spectacular results. Birds in the control group weighed 1200 g, while those fed the T-2 toxin containing feeedstuff weighed 500 g at 8 weeks of age. This means more feedstuff, time and cost to achieve a given body weight (Bitay et al., 1979). We studied the effect of T-2 toxin on laying hens. Egg production dropped within 4–6 days after the uptake of toxin-containing feed. The effect depended on the amount of toxin ingested. After toxin feeding had been stopped, egg production gradually returned to the normal level, except when the quantity of the toxin fed had been high.

The metabolism of *T-2 toxin* and *diacetoxyscirpenol* in hen's egg was also studied. A 5 microgram toxin dose was inoculated into the yolk sac of fertile hen's eggs, then the eggs were incubated and analysed for the presence of metabolites up to the 11th day. T-2 toxin was transformed into HT-2 toxin, T-2 triol, neosolaniol, deacetyl-neosolaniol and T-2 tetraol. Diacetoxyscirpenol was changed into monoacetoxyscirpenol, then to scirpentriol (Bata et al., 1983*b*).

The metabolism of trichothecenes was investigated in rumen fluid *in vitro*. The rumen fluid, containing bacteria and protozoans, decomposes the toxins in 3 to 4 h. This experimental result corresponds to our observations that adult ruminants are practically resistant to the effect of trichothecenes. These toxins cause illness only in young animals in which the rumen microflora has not developed yet, or in fully grown animals in which digestion in the rumen is disturbed.

Dankó's research on *stachybotryotoxicosis*, his pathological descriptions and immunological results raised international attention. Professor Mirocha named him "the leading living authority on stachybotryotoxicosis". Dankó concluded that the toxin produced by *Stachybotrys alternans* hindered immunological reactions by suppressing the formation on immunoglobulins (Dankó and Tanyi, 1968; Dankó, 1972; Dankó, 1976).

For some time stachybotryotoxicosis has often been diagnosed in Hungary (Harrach et al., 1987; Sándor, 1984).

In recent years the pathological changes induced by *T-2 toxin* and *satratoxin H* in the lymphoid organs of mice have been studied. These toxins caused similar lymphocyte depletion and necrosis in the B and T dependent zones of the lymphoid organs. The severity of the lesions was dose dependent. Satratoxin H proved five times as toxic as T-2 toxin (Glávits and Ványi, 1988).

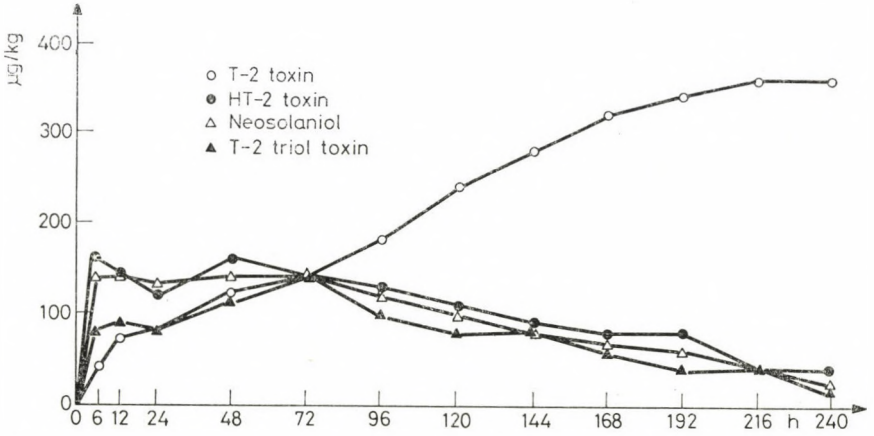

Fig. 8. Toxin concentration of the liver (µg/kg) in rabbits treated with 1 mg T-2 toxin/kg body weight daily

There is an intensive rabbit breeding in Hungary, so the pathology, metabolism and excretion of T-2 toxin were investigated also in this species. Toxin doses of 4 ppm and higher caused death within 48 h. The pathological examination revealed acute gastritis, enteritis, necrosis of the intestinal mucosa and necrosis in the liver, lymphoid tissues and bone marrow. In the subacute toxicological experiment loss of weight and similar but milder changes were observed. In the course of toxin consumption the metabolizing capacity of the liver decreases and sensitivity of the organism increases (Fig. 8).

Trichothecenes impair the protective ability of the mucous membranes locally and that of the whole organism in general. As a result, various bacteria, viruses and parasites can cause severe disease. For example, we observed acute coccidiosis in chickens although the feed contained coccidiostatic substances in an effective concentration but also T-2 toxin.

To investigate this interaction an experiment was carried out on broiler chickens (Table I).

The presence of T-2 toxin aggravates coccidiosis and in such cases monensin cannot hinder the development of the disease.

In Hungary feed refusal and vomiting have been observed among pigs for many years. We succesfully elucidated the cause of this and isolated *deoxynivalenol* first in 1981. In 1985, the year of a serious *Fusarium* infection, a country-wide survey established that 82.5% of the feeds were contaminated with deoxynivalenol (Sándor et al., 1988).

In an experiment carried out with 384 pigs deoxynivalenol not only decreased the feed consumption and body weight gain but also predisposed

Table I

Interaction between T-2 toxin and coccidiosis

Groups	Number of animals	Treatment			Death	SS	Weight gain			Gross production %
		I	M	T			Total	Average (g)	%	
1	10	+	+	+	2	++	2050	342	81	48.6
2	10	+	+	0	—	+	2900	362	85.8	68.7
3	10	+	0	+	7	+++	470	235	55.6	11.1
4	10	+	0	0	3	++	1800	360	85.3	42.6
5	10	0	+	+	—	—	3950	395	93.6	93.6
6	10	0	+	0	—	—	4230	423	100	100
7	10	0	0	+	—	—	4300	430	102	102
8	10	0	0	0	—	—	4220	422	100	100

I = infection with coccidium oocysts (6 million/chicken)
M = addition of monensin (100 mg/kg feedstuff)
T = addition of T-2 toxin (6 mg/kg feedstuff)
SS = severity of symptoms

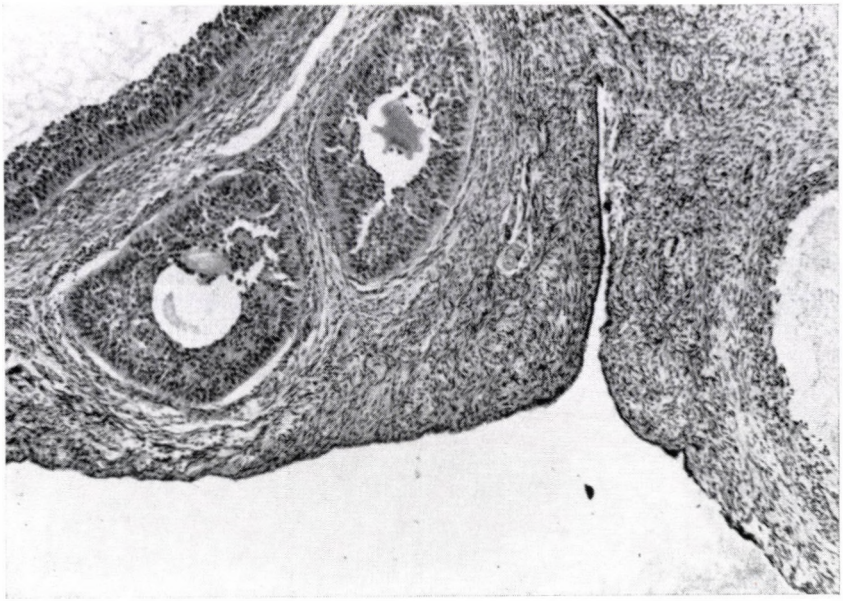

Fig. 1. Degenerated ovum in ovary from a sow treated with zearalenone. Haematoxylin and eosin, × 250

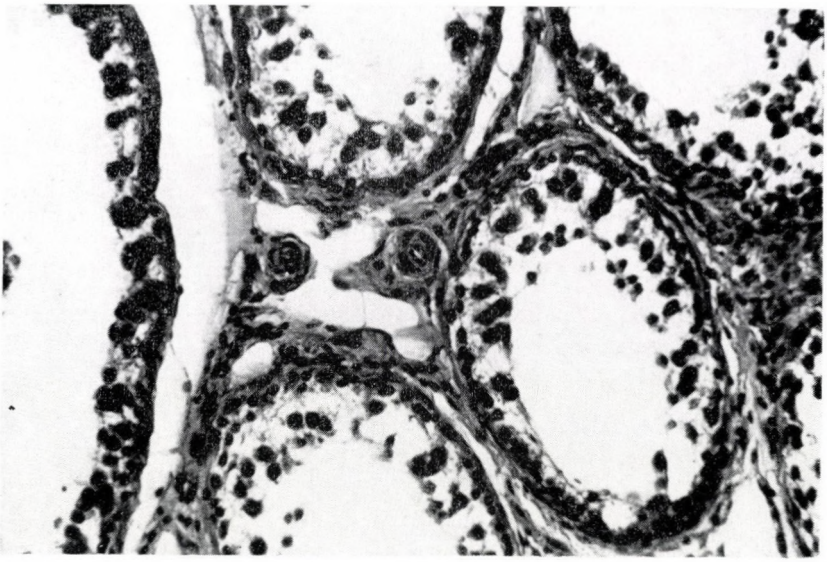

Fig. 2. Histopathological section of testicle from a ram consuming zearalenone-containing feed (field case). The germinal epithelium is nearly destroyed. H. and E., × 250

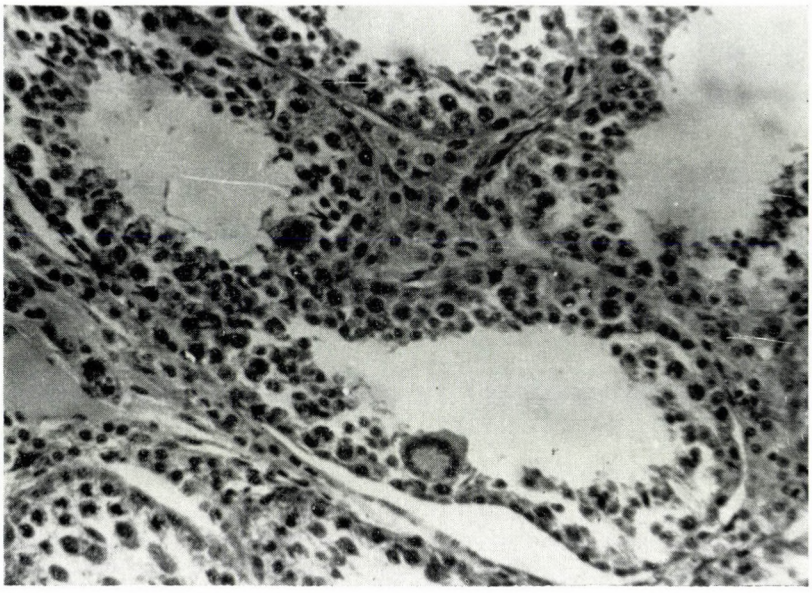

Fig. 3. Histopathological section of testicle from a boar treated with zearalenone. H. and E., × 250

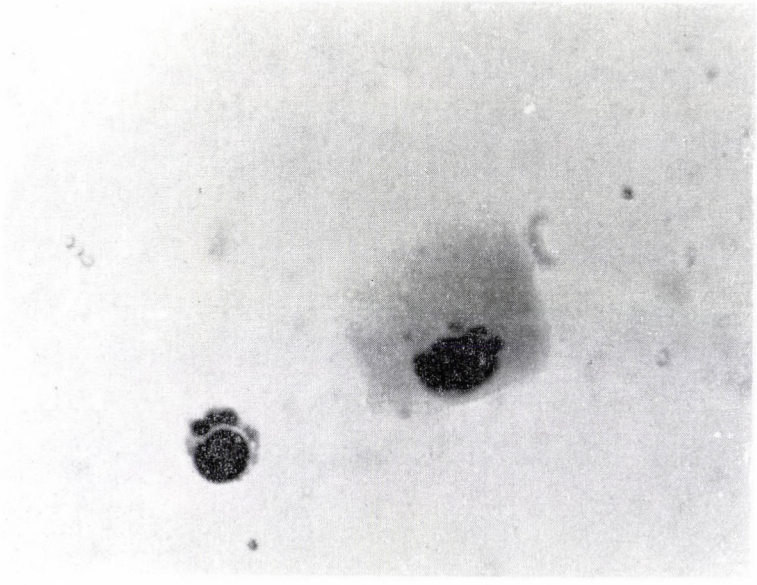

Fig. 4. Monkey testicle monolayer cell culture in the 16th h after treatment with zearalenone. H. and E., × 1,400

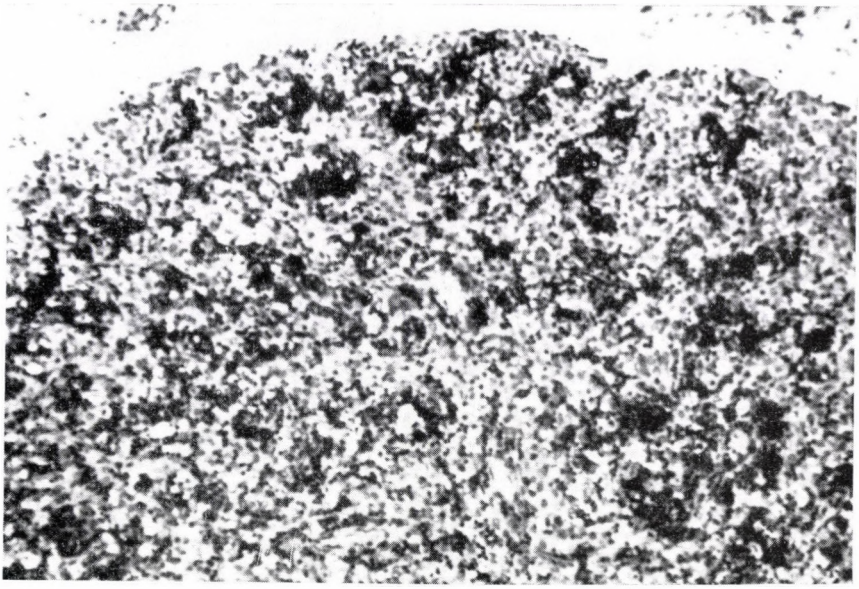

Fig. 5. Part of lymph node from a mouse treated with 1.6 mg of satratoxin H/kg body weight. The mouse died 24 h after treatment. H. and E., × 160

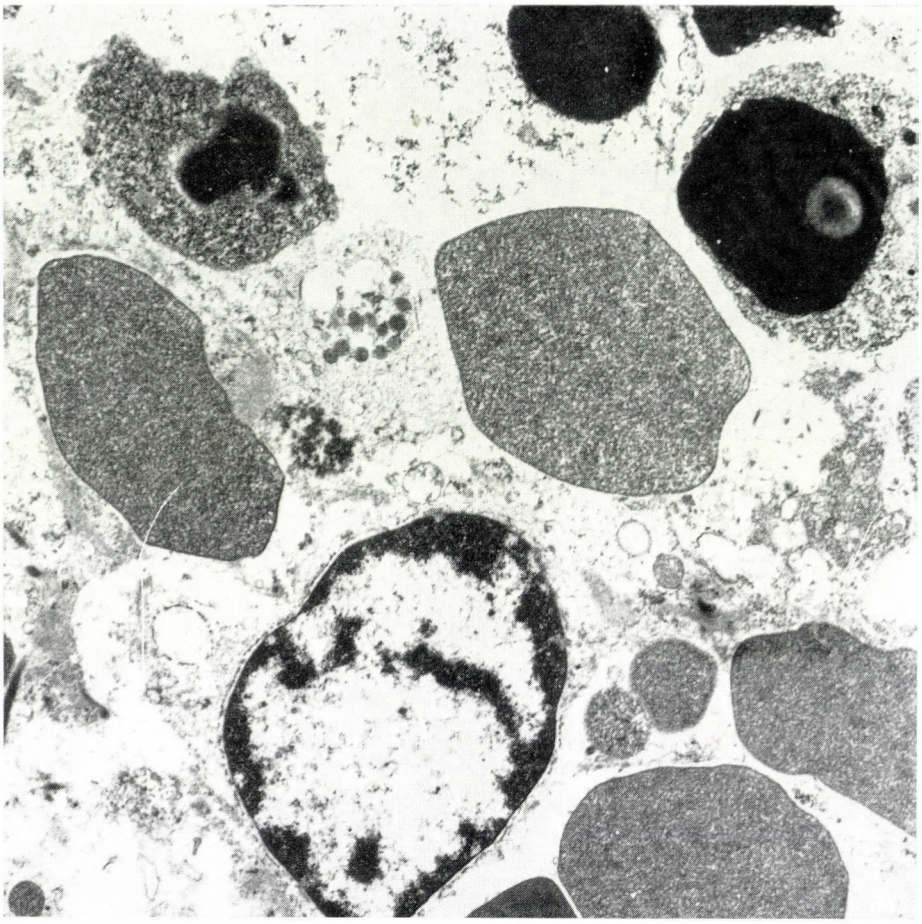

Fig. 6. Part of spleen from a mouse treated with 1.75 mg satratoxin H/kg body mass. The mouse died 24 h after treatment. Electron micrograph, × 9,600

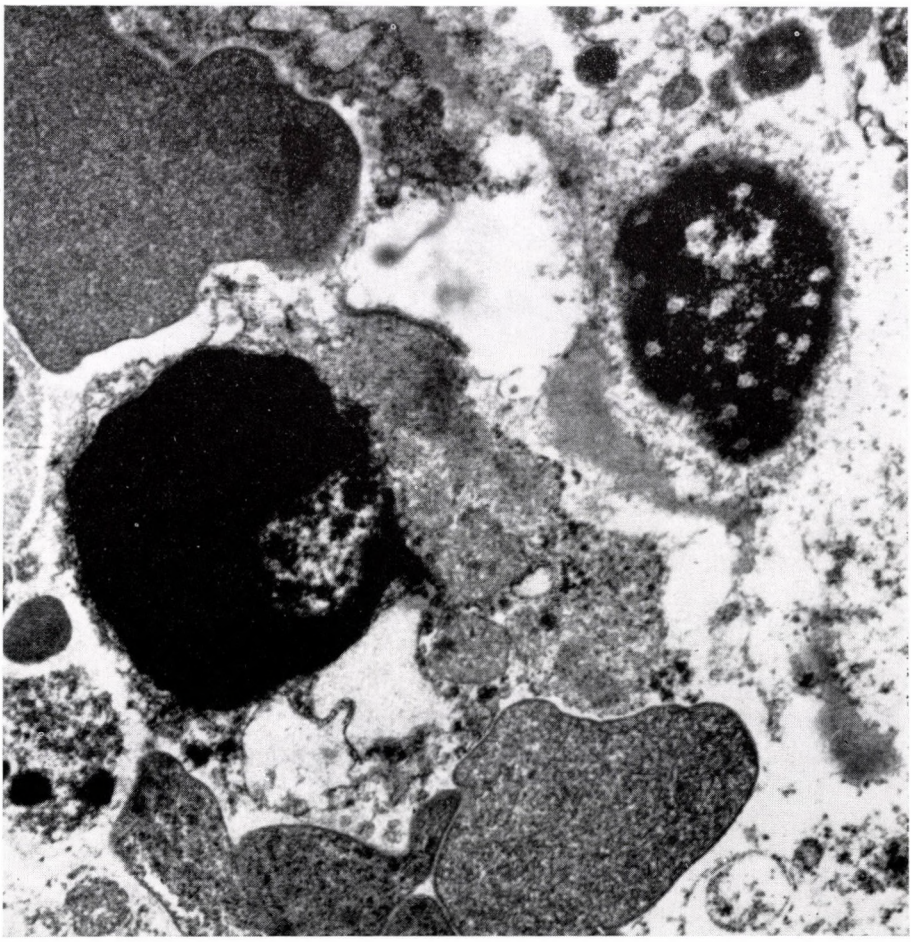

Fig. 7. Part of the mesenteric lymph node from a mouse treated with 4 mg T-2 toxin/kg body weight i.p. and killed 48 h after treatment. Electron micrograph, × 14,300

Table II

Effect of deoxynivalenol on pig fattening

	Experimental group	Control group
Number of piglets	384	384
Average daily weight gain, g/day	485	506
Feed used up for 1 kg weight gain, kg	3.82	3.59
Cost of diet for 1 kg weight gain, Ft	21	19
Cost of pharmaceuticals, Ft	25,721	3641
Total income, million Forints (duration of experiment: 31 days)	1.16	1.32
Toxin content of the diet: 1500 μg/kg		

the animals to dysentery. In the toxin-treated group treatment with pharmaceuticals remained ineffective (Ványi and Sándor, 1988; Table II).

We conducted feeding trials also with *ochratoxin A* and *patulin* in broiler chickens throughout 6 weeks. Two ppm ochratoxin A in the feed reduced body weight gain by 300 g, while 5.6 ppm patulin in the feed resulted in 450 g shortage in gain. Considering that the average size of large chicken flocks is 100–150 thousand, on such farms the economic losses may be huge (Bitay et al., 1979).

Because of the contamination of feeds with ochratoxin A it was suspected that mycotoxic nephropathy of swine diagnosed in Scandinavian countries might occur also in Hungary. We conducted an extensive two-year survey including 4 slaughterhouses (1.5 million slaughtered pigs per year). 227 kidney samples showing gross lesions were submitted to our laboratory from these slaughterhouses for chemical and histopathological analysis. 26.9% of the samples were positive with a toxin content of 5–50 ppb. In 5 cases the toxin content exceeded 100 ppb (Sándor et al., 1982).

Ten to twelve years ago we investigated the deterioration of quality of grains caused by storage moulds. By microbiological examination of 2790 samples we found that 38–42% of the mould flora consisted of aspergilli and 21–24% were penicillia. We analysed the toxin-producing capacity of 100 *Aspergillus* and 100 *Penicillium* strains. Nineteen *Aspergillus* strains and 29 *Penicillium* strains produced various mycotoxins (Sellyey-Sándor, 1978; Szailer and Sellyey-Sándor, 1977). We studied the influence of time on toxin production and the effect of heat treatment on storage moulds (Sándor, 1986).

We conducted experiments aiming the *decontamination* of feedstuffs from mycotoxins. We applied low level gamma irradiation known as a routine technique in the food industry. We investigated the effect of irradiation on *Fusarium*, *Aspergillus* and *Penicillium* strains, using 0.5 to 1.5 Mrad and

Table III

Mycotoxins in moist-stored corn

	Aspergillus and *Penicillium* toxins	*Fusarium* toxins
Number of samples	170	66
Number of toxin-containing samples*	46 (27%)	25 (37.8%)
Detected toxins:		
ochratoxin A	41	—
citrinin	13	—
patulin	3	—
zearalenone	—	8
T-2 toxin	—	13
HT-2 toxin	—	5
T-2 tetraol	—	6
acetyl T-2	—	3
diacetoxyscirpenol	—	9
deoxynivalenol	—	2

* Some samples contained more than one toxin

20–500 krad/h output irradiation. The strains' growth ability did not cease, it only decreased a little. The toxin-producing capacity of the surviving strains increased 2 to 5 times (Sándor et al., 1980).

The *moist storage* of corn is becoming more and more widespread in Hungary. It seemed reasonable to survey the toxin contamination of this moist corn (Table III). Zearalenone, T-2 toxin and its metabolites, diacetoxyscirpenol and deoxynivalenol, were found in a quantity of 50 to 500 μg/kg of feed. The quantity of zearalenone increased during storage, the quantity of T-2 toxin decreased slightly and at the same time the metabolites appeared (Sándor et al., 1984*a*; Sándor et al., 1984*b*).

In our laboratory we have performed *mycotoxin analyses* first of all from feed samples for the practice since 1973. During this period we collected the clinical symptoms, pathological lesions and production disorders related to the results of mycological examination of feeds and of the toxin analyses. Part of our results has been published (Sándor et al., 1984). On the average we analyse 1500 samples a year, with a positivity rate of 15 to 20% (Table IV). Of course, this does not mean such a high contamination of the Hungarian feedstuffs in general, as our samples were collected in connection with animal health problems.

We analyse 12 or 13 kinds of mycotoxins routinely and some others for research purposes only. The analytical methods are elaborated by us or we adapt those from the literature and modify them corresponding to our pos-

Table IV

Mycotoxins detected between 1977 and 1987

Mycotoxins	Number of analyses	Positive results	
		n	%
Zearalenone	2515	263	10.46
T-2 toxin	6081	1395	22.94
Diacetoxyscirpenol	1633	185	11.33
Deoxynivalenol	941	298	31.67
Satratoxins	62	12	19.36
Ochratoxin A	2498	411	16.45
Rubratoxin B	385	29	7.53
Citrinin	374	20	5.35
Patulin	619	37	5.98
Aflatoxins	275	11	4.00
Other toxins	467	84	17.99

sibilities (Bata et al., 1983*a*; Sándor, 1982; Ványi et al., 1985). We use thin-layer chromatography, capillary gas chromatography, fluorometry, and now we are introducing into our work high-performance liquid chromatography.

In 1978, the President of the Hungarian Academy of Sciences awarded us the grand prize of agricultural sciences for the results of mycotoxin research.

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ELECTRON MICROSCOPIC STUDY OF THE PERITONEAL MACROPHAGES OF RATS WITH CHRONIC FASCIOLIASIS AND THE CARCINOGENIC EFFECT OF DIETHYLNITROSAMINE

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The ultrastructure of peritoneal macrophages of rats with chronic fascioliasis and the carcinogenic effect of diethylnitrosamine (DENA) were studied. The phagocytic activity of macrophages on *Staphylococcus aureus* was examined in its dynamics (in the 1st, 5th and 24th h). The ultrastructural changes of the macrophages were the most pronounced in animals injected eight times with DENA and the weakest in the group of animals infected twice with *Fasciola hepatica.*

As to the phagocytic activity of the macrophages the following events were observed: attraction and adhesion of the bacterial cells to the surface of the macrophages, their inclusion in phagosomes; partial to full lysis of the bacteria; and formation of residual bodies. The phagocytosis was the most active in macrophages obtained from animals infected twice with *Fasciola hepatica* and the weakest in those from the DENA-treated animals.

Keywords: Macrophages, chronic fascioliasis, carcinogenesis, rat

The problem of interaction between helminth invasion and cancer is relatively new and poorly studied. It is of special interest because of the increasing environmental contamination with chemical carcinogens and the considerable frequency of parasite invasions. So far only the combined effects of nitrosamines and schistosome invasion have been studied in relation to cancer, viz. bladder cancer (El-Merzabani et al., 1979; El-Aazer et al., 1979; Marian, 1982) and those of nitrosamines and opisthorchosis on experimentally induced opisthorcho-cholangiocarcinoma (Thamavit et al., 1978; Flavel, 1981; Flavel and Lucas, 1982, 1983). Similar investigations of other helminthiases, including fascioliasis, have not been done.

In recent years we have studied the interaction between helminth invasion *(Fasciola hepatica)* and chemical carcinogenesis (caused by diethylnitrosamine, DENA) in the liver of rats under different experimental conditions (Tsocheva, 1986; Krustev et al., 1987; Tsocheva et al., 1987).

Based on the results, a hypothesis has been suggested for the participation of the specific and nonspecific reactivity of the organism in the pathogenesis of this interaction (Tsocheva et al., 1989). In these processes cell

populations not only of the liver but also those of other units of the immune system participate. In this respect it seemed to be of interest to trace out the changes in the structure and functional activity of peritoneal macrophages.

We have considered the morphology and functional activity of rat peritoneal macrophages under the effect of *Fasciola hepatica* and DENA in a number of studies (Mizinska-Boevska et al., 1988; Mizinska-Boevska et al., 1989*a*; Mizinska-Boevska et al., 1989*b*). Shortly after DENA administration only toxic effects are exerted in the liver, carcinogenic properties appear after a long latent period. In a previous paper (Mizinska-Boevska et al., 1989*b*) we investigated the toxic effects of DENA on peritoneal macrophages in chronic fascioliasis.

In the present paper the structural and functional changes of rat peritoneal macrophages were studied in chronic fascioliasis during the carcinogenic action of DENA.

Materials and methods

Male white Wistar rats aged 30–35 days, 80–100 g in body mass, were used. The animals were divided in five groups as follows.

Group I: controls (animals neither infected nor treated with DENA).

Group II: animals twice infected (on the 1st day and in the 10th week) with *F. hepatica*.

Group III: animals injected with DENA from the 6th week on, 8 times at 7-day intervals.

Group IV: animals infected with *F. hepatica* once and injected with DENA 8 times from the 6th week on.

Group V: animals infected with *F. hepatica* twice and injected with DENA 8 times from the 6th week on.

Each group contained 12 animals. All were decapitated in the 27th week of the experiment.

Each of the rats was orally infected with 15 adolescariae of *F. hepatica* (Polyakova-Krusteva, 1970). DENA, 100 mg/kg body mass, was injected intraperitoneally (Bikorez and Pinchuk, 1976).

The ultrastructure of the macrophages isolated from the peritoneum of the experimental animals was studied. For establishing their functional state, their phagocytic activity on *Staphylococcus aureus* was tested *in vivo* throughout. The scheme of application of the bacteria and isolation of the peritoneal macrophages was described by us in detail earlier (Mizinska-Boevska et al., 1988).

Suspension of peritoneal macrophages was processed after the standard scheme for electron microscopic studies: fixation in 4% glutaraldehyde and

then in 1% OsO_4, and embedding in Durcupan. The ultrathin sections were cut in Ultratom LKB III and, after being contrasted with uranyl acetate and lead citrate, were examined in an Opton 10 C electron microscope.

Results

The ultrastructure of the macrophages and their phagocytic activity were studied in their dynamics in the 1st, 5th and 24th h.

I. *Controls*

The peritoneal macrophages from the control group were round. Their surface had the following characteristics: from almost smooth (in the 1st h) to strongly folded with a large number of projections, pseudopodia (in the 24th h).

Ultrastructure. The nuclei were large, round or kidney shaped.

The chromatin was localized at the periphery of the nucleus, in the inner layer of the nuclear membrane. In the cytoplasm, the main types of cell organelles were demonstrable.

Mitochondria as well as primary and secondary lysosomes were normal in shape and size. The endoplasmic reticulum was represented mainly by its rough form (RER).

Phagocytosis. In the 1st h after applying *S. aureus*, the bacterial cells were attracted and adhered to the surface of the macrophages (Fig. 1); bacteria were observed in the cytoplasm of most of the macrophages. The phagocytosed bacteria, single or more in number, were included in phagosomes, which often formed heterophagolysosomes with primary lysosomes (Fig. 2). In the 5th h heterophagosomes whose periphery was filled with numerous parallel membranes and myeline figures, appeared as new elements (Fig. 3). In the 24th h the phagocytosed bacteria showed different stages of destruction: from partial to full lysis and formation of residual bodies (Fig. 4).

II. *Animals with twofold F. hepatica infection*

Ultrastructure. The ultrastructure of the macrophages from Group II was quite similar to that of the control cells. The following specificities may be pointed out: from the 1st to the 24th after applying staphylococci the cell surface showed signs of activation. Here, however, already in the 5th h numerous pseudopodia appeared. These tended to fold and be incorporated in the cytoplasm, and formed small cavities (surface vacuoles), which were often seen in groups of 2 to 11 (Fig. 5). The nucleus, the mitochondria, the lysosomes and the RER did not differ morphologically from the control forma-

tions. In the macrophages isolated in the 5th h, elements of the Golgi apparatus, and in their vicinity parallel tubes and small vacuoles were observed (Fig. 6).

Phagocytosis. In the 1st h phagosomes with a single bacterium or a group of bacteria in them, were observed in the cytoplasm (Fig. 7). Also, single residual bodies appeared (Fig. 8), i.e. the process of phagocytosis seemed to be somewhat accelerated in comparison to the controls. In the 5th h heterophagosomes similar to those decribed in the control group were observed with myeline figures and bacteria at different stages of lysis. Intact bacterial cells were demonstrated neither at 5 h nor at 24 h.

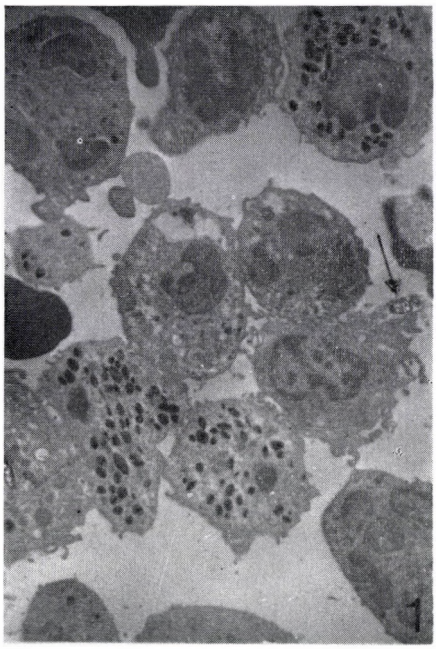
III. *DENA-treated animals*

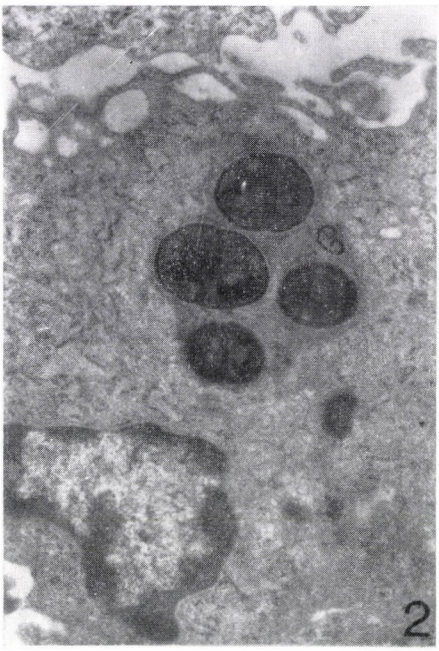
Ultrastructure. Macrophage ultrastructure was seriously affected. Changes were observed in all cell organelles. In separate macrophages the nuclei were of low electron density and poor in chromatin, which outlined the contours of the nucleus as a thin line (Fig. 9). An increase in the number of pores in the nuclear membrane and nuclear bodies were demonstrated (Figs 9 and 10). Nuclear bodies are spherical formations filled with granules of filamentous elements situated in the karyoplasm. The mitochondria tended to increase in size. The matrix got lighter in colour and their cristae were strongly reduced, i.e. the mitochondria were swollen (Fig. 11). A higher number of lysosomes in all their forms were observed. The RER consisted of short and wide cisterns.

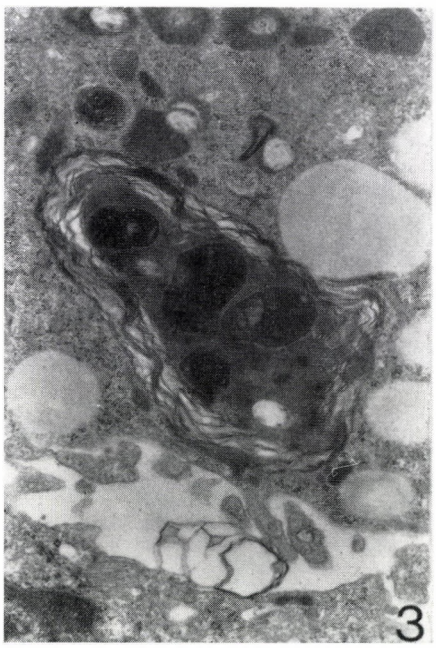
Phagocytosis. The phagocytosis follows the scheme described for the controls: in the 1st h single or grouped bacteria were observed in phagosomes (Fig. 12). In the 24th h phagosomes with myeline figures appeared (Fig. 13) as well as residual bodies in which the included material was at different stages of lysis (Fig. 14).

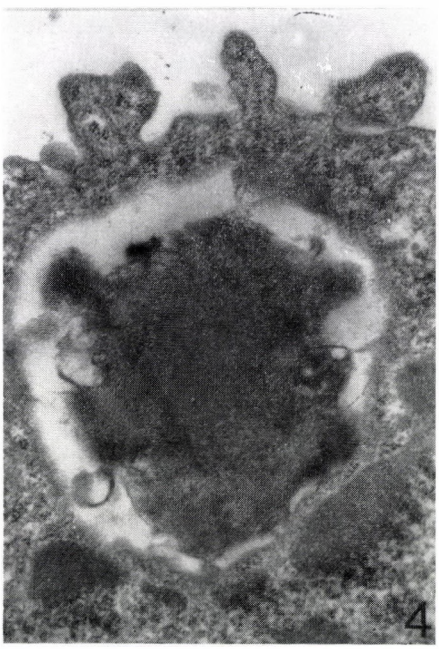
IV. *F. hepatica-infected and DENA-treated animals*

Ultrastructure. The ultrastructure of the macrophages was preserved. The cytoplasm appeared highly vacuolated as early as in the 1st h after staphylococcus had been added (Fig. 15). In the 5th h well-expressed vacuoles were not observed but some zones irregular in shape appeared which were lighter in colour than the rest of the cytoplasm and were filled with a fibrous substance and destroyed cell organelles (Fig. 16). These "light" zones in the cytoplasm were not surrounded by membrane and thus they cannot be considered residual bodies or digestive vacuoles (Fig. 17). By the 24th h the cytoplasmic structure was normalized, the vacuoles and the destructive zones disappeared. The main types of cell organelle morphology were similar to those in the control.

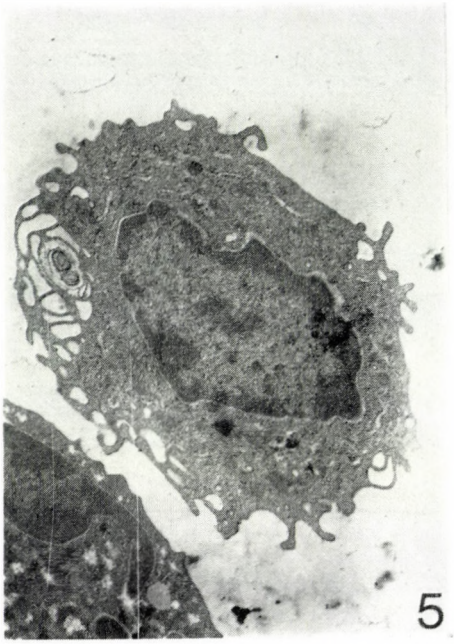


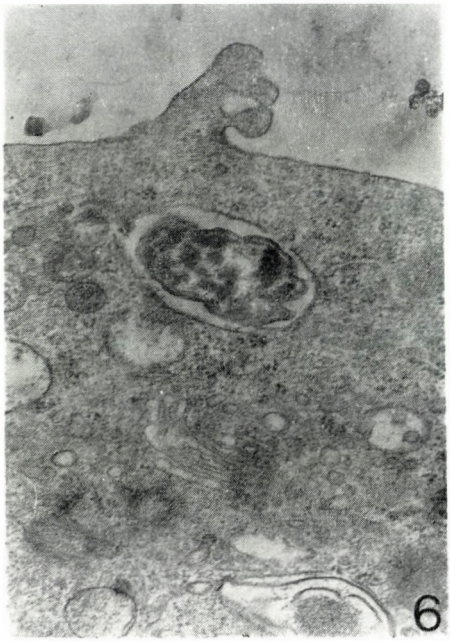


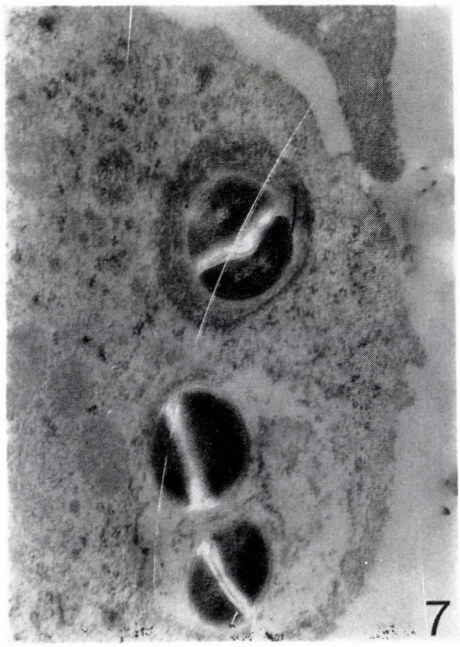


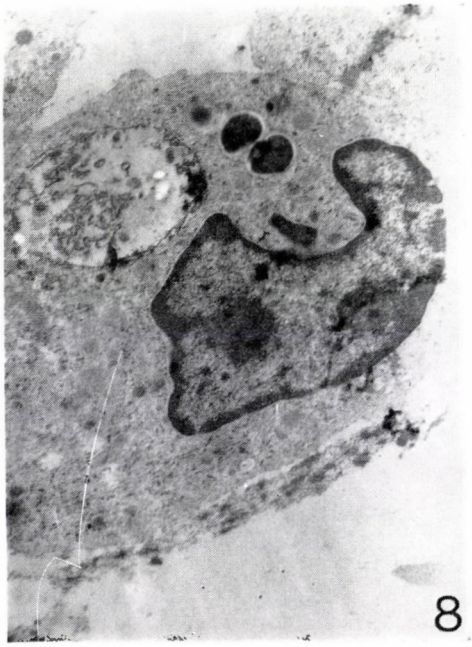


Figs 1–4. Controls. *1:* General view on the morphology of peritoneal macrophages. Bacterial cell adherent to the surface (arrow). × 2,500. *2:* Phagosome with bacterial cells in it. × 12,500. *3:* Heterophagosome with bacteria and myeline figures. × 12,500. *4:* Residual body filled with a mass of cell debris and bacteria. × 7,000

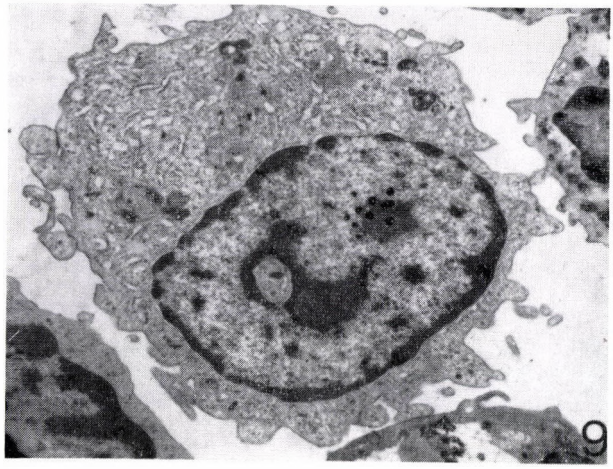








Figs 5–8. Animals infected with *F. hepatica* twice. *5:* Surface-situated vacuoles. × 8,000. *6:* Elements of the Golgi apparatus. × 25,000. *7:* Phagosomes with single or several bacteria. × 16,000. *8:* Residual body in the cytoplasm of a macrophage. × 6,300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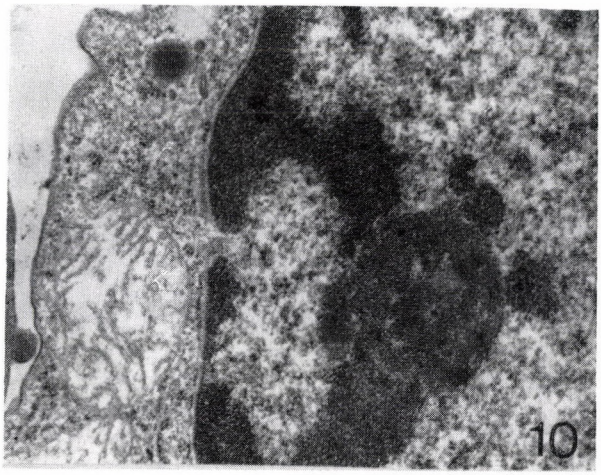
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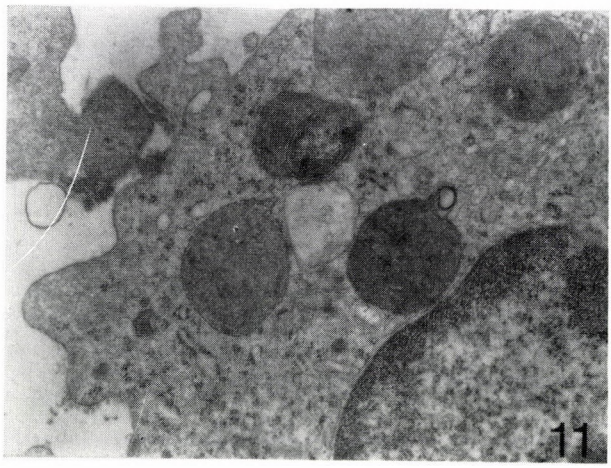
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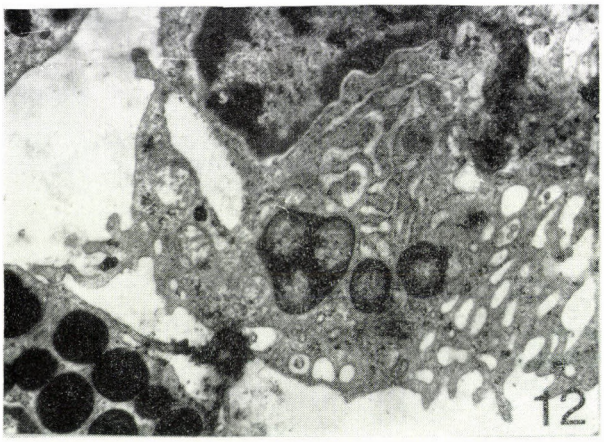
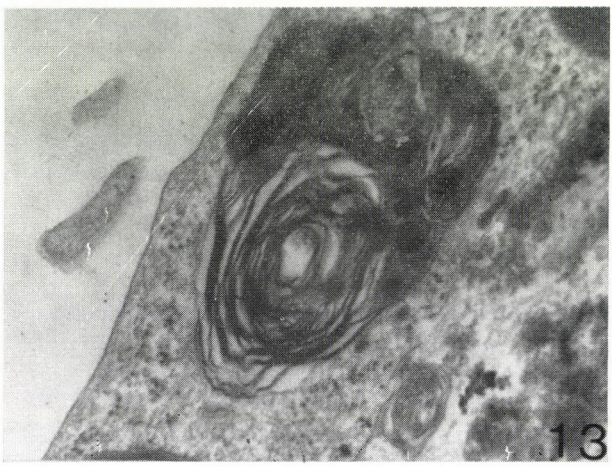
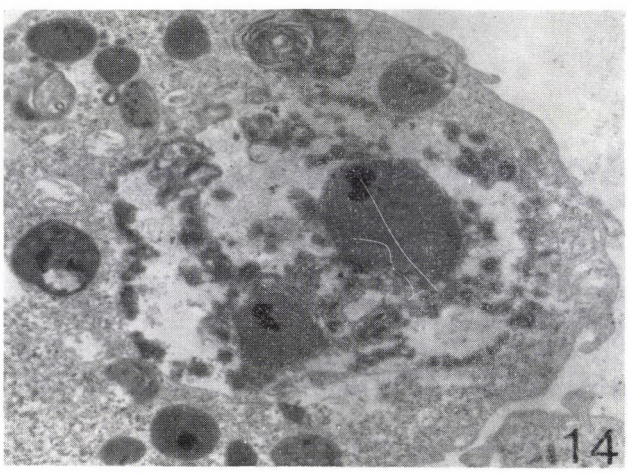
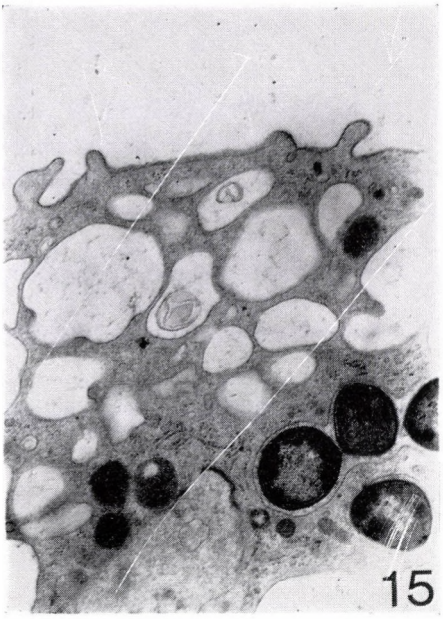
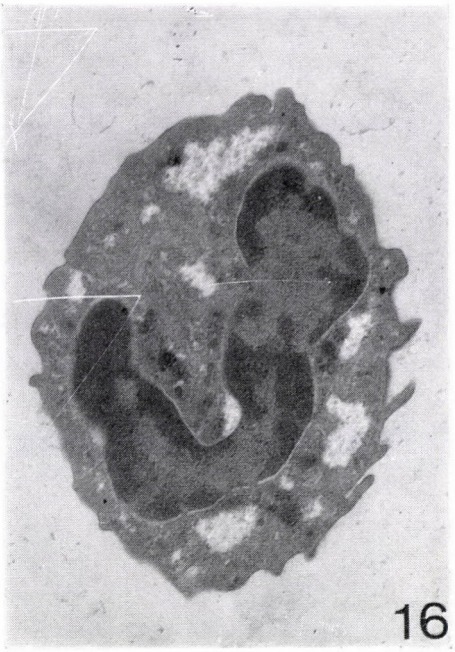


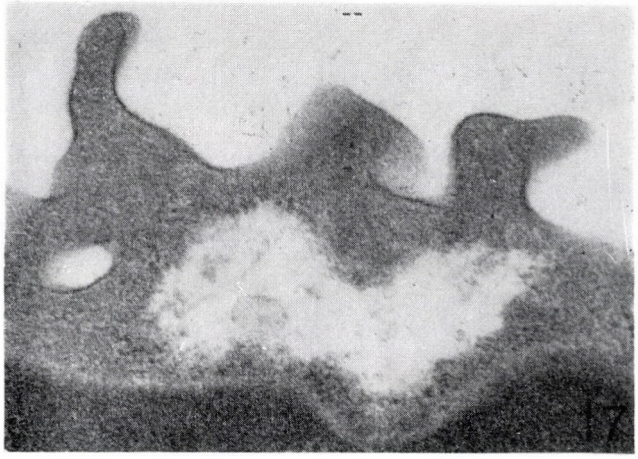


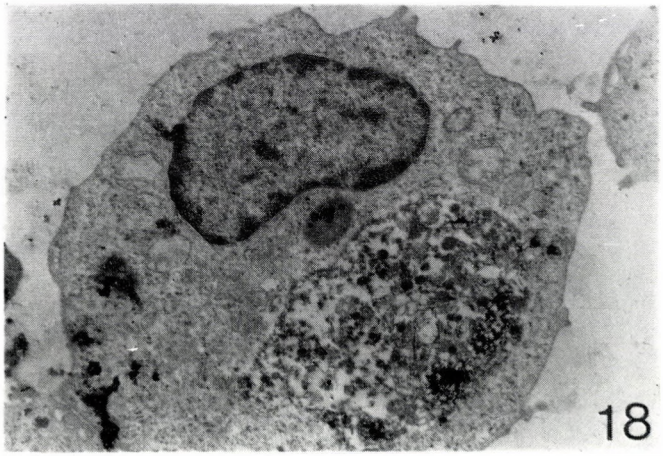


Figs 9–14. DENA-treated animals. *9:* Nucleus with a large number of pores and a nuclear body. × 6,300. *10:* Detail of Fig. 9. × 16,000. *11:* Swelling of mitochondria. × 12,500. *12:* Bacterial cells in the cytoplasm of macrophages. × 40,000. *13:* Phagosome with bacteria and myeline figures × 25,000. *14:* Residual body with material at different stages of bacteriolyis. × 80,000

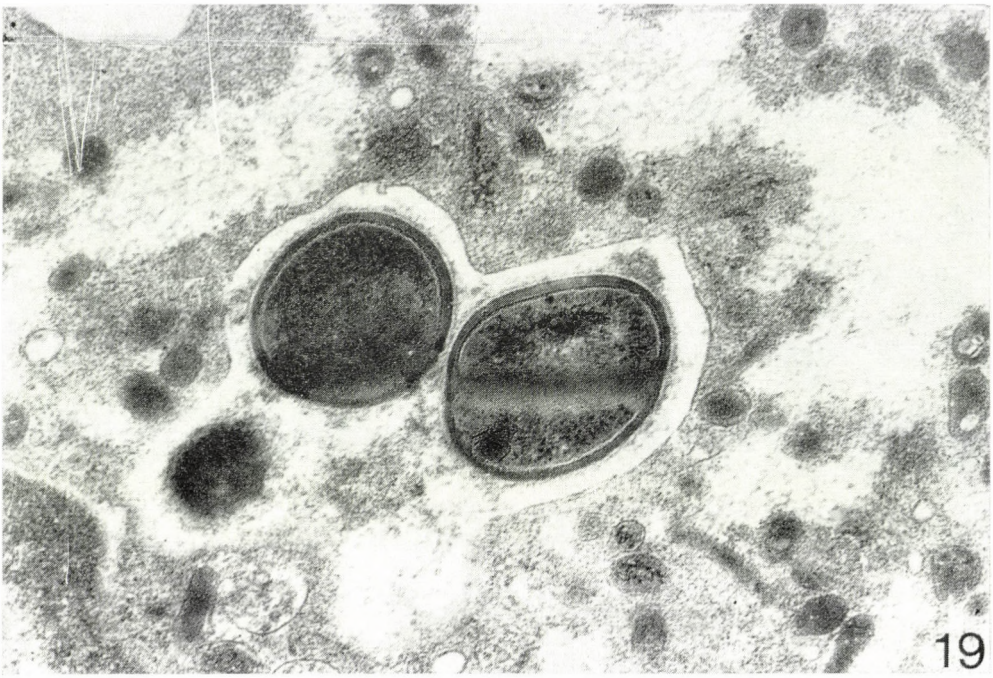


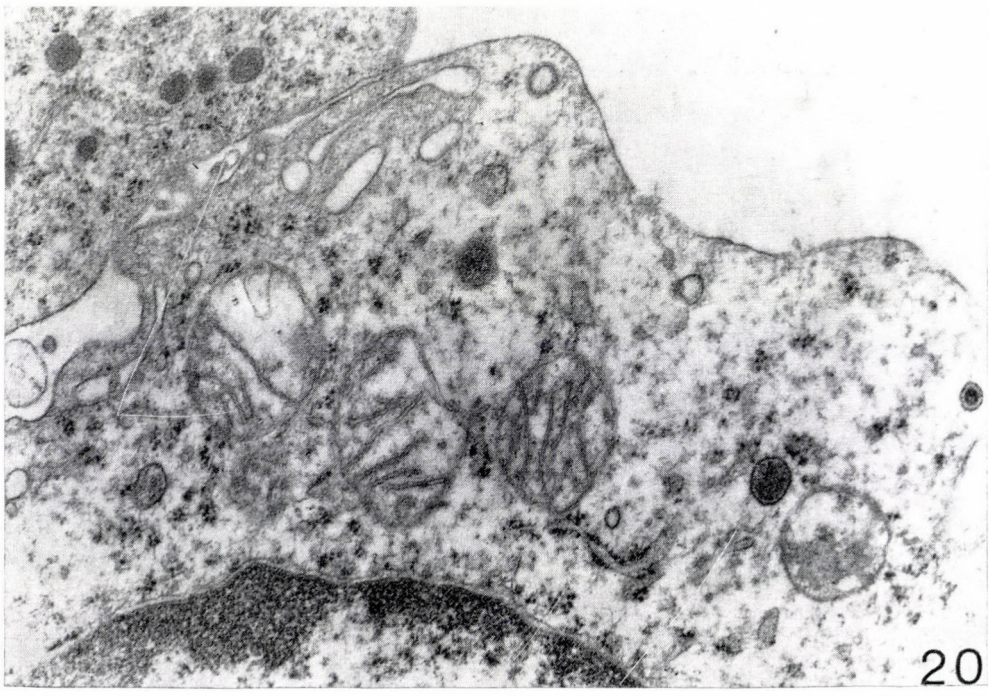






Figs 15–18. Animals infected with *F. hepatica* once and treated with DENA. *15:* Strong vacuolization of the cytoplasm, and phagosome with bacteria. × 12,500. *16:* "Light" zones in the cytoplasm. × 8,000. *17:* Detail of Fig. 16. × 40,000. *18:* Digestive vacuoles. × 5,000





Figs 19–20. Animals infected with *F. hepatica* twice and treated with DENA. *19:* Zones in the cytoplasm and phagosomes with bacteria. × 20,000. *20:* Mitochondria and elements of RER in the cytoplasm. × 12,500

Phagocytosis. The phagocytosis appeared similar to that in the controls: phagosomes with one or several staphylococcus cells in them were seen in the 1st h (Fig. 15) and typical digestive vacuoles in the 24th h (Fig. 18).

V. *Animals infected twice with F. hepatica and treated with DENA*

The morphology of the macrophages in this group is similar to that described for group IV. Here, however, besides vacuolization of the cytoplasm, zones irregular in shape with no limiting membrane appeared already in the 1st h; the zones contained a net-like fibrillar substance (Fig. 19). The ultrastructure of the nuclei, mitochondria, lysosomes and RER was similar to that described in the control group (Fig. 20).

The phagocytosis follows the above-described scheme, viz. phagosomes (Fig. 19), heterophagosomes with myeline figures, and residual bodies appeared in the 1st, 5th and 24th h, respectively.

Discussion

The changes in the ultrastructure of macrophages were the most serious in animals injected 8 times with DENA. Despite the fact that by the 27th week of the experiment the direct toxic effect of DENA had decreased, its carcinogenic effect on the host (the liver in particular) was well expressed. This affected the structure of the peritoneal macrophages as well, whereby mainly the nuclei and the mitochondria were involved in the pathogenic process. The observed swelling of the mitochondria is a proof for changes in the absorption of oxygen as well as in oxidoreductase activity (Krustev et al., 1982). The nuclear pores were increased in number and nuclear bodies appeared.

In the animals infected with *F. hepatica* the structure of the macrophages was almost unchanged and quite similar to that of the controls.

Under the combined effect of both pathogenic factors (groups IV and V) only ultrastructural changes were demonstrated, viz. intense vacuolization and appearance of necrotic zones in the cytoplasm of macrophages.

As to the phagocytic activity of the macrophages the picture in its dynamics was similar to that in the control group in all experimental groups. The following order in the process of phagocytosis can be outlined: incorporation of bacterial cells in phagosomes in the cytoplasm of the macrophages, partial to full lysis of the bacteria, and formation of residual bodies. Among all the five groups, very slight differences were noticed in the phagocytic activity of the peritoneal macrophages. The macrophages from group II (twofold *F. hepatica* infection) were the most active and those in group III (rats

treated with DENA) were the least active. Under the combined effect of fasciola infection and DENA (groups IV and V) the functional activity was more intense than in the control group. Therefore, our morphological results agree with the results obtained in the microbiological study published by Dimov et al. (1985) in which the phagocytic activity was characterized by the following values: group II: 49.4 ± 8.5; group III: 21.3 ± 4.1; group IV: 40.2 ± 6.1; group V: 36.5 ± 3.6; and 29.1 ± 4.6 for the control group.

In conclusion, we established that when the two factors are applied separately, the behaviour (structure and function) of the macrophages is entirely different, ranging from normal (under helminth infection) to strongly changed (under the effect of the chemical carcinogen DENA). Under the combined effect of both pathogenic agents, however, the changes are moderate, i.e. the two factors seem to counterbalance each other's influence.

The two pathogenic factors (*F. hepatica* and DENA) applied either separately or in combination affect mainly the structure and less markedly the functional activity of the macrophages as tested at a late stage (27th week) of the experiment. The observed changes prove that the process is not restricted to the liver. In the immune response of the host further units of the immune system, peritoneal macrophages among them, take part.

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NEW ASPECTS OF THYROID PHYSIOLOGY: A REVIEW

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1. Thyroid hormone economy in general

Thyroid hormones are responsible for normal metabolism in the adult and for the normal development of the central nervous system in the neonate. Besides these two main roles these hormones influence almost all catabolic and anabolic processes in the organism. All of the cells of the body are known to be responsive to thyroid hormones (even if this response might not involve increased oxygen consumption in some tissues). The particular response, however, may vary fundamentally according to the type of tissue.

The research efforts of the past decade have brought a tremendous amount of information concerning the regulation of thyroid hormone action and metabolism. One of the most important recognitions is that organs other than the thyroid gland can import thyroid hormones from the blood pool not only in order to utilize them for their own purposes but also in order to activate them and redirect the activated forms into the blood pool so that other organs and cells can make use of these activated forms. Therefore, it is likely that the cells themselves which activate and utilize thyroid hormones must be considered as fundamental parts of the whole regulatory mechanism.

The final thyroid hormone action depends on the availability of the hormones for the cells and on the responsiveness of these cells to thyroid hormones. The old concept of hierarchical relationships among the central nervous system (CNS), the hypothalamus (HT), the pituitary (HP), the thyroid gland (TH) and the peripheral cells (PC) forming a unidirectionally regulated system does not prevail any more. Rather a dynamic syncytial way of interrelationship can be postulated, where the PC is an actively regulating part of the system by (1) calling for hormone when in need of that, (2) activating and inactivating thyroid hormones entering the cell, and (3) displaying enhanced or decreased sensitivity to the hormone by altering the status of the second messenger system within the cell.

This complex regulatory mechanism is modulated by the distribution of the hormones and by other endocrine and neural factors. These various effects, all of which can be influenced by exogenous physical or chemical

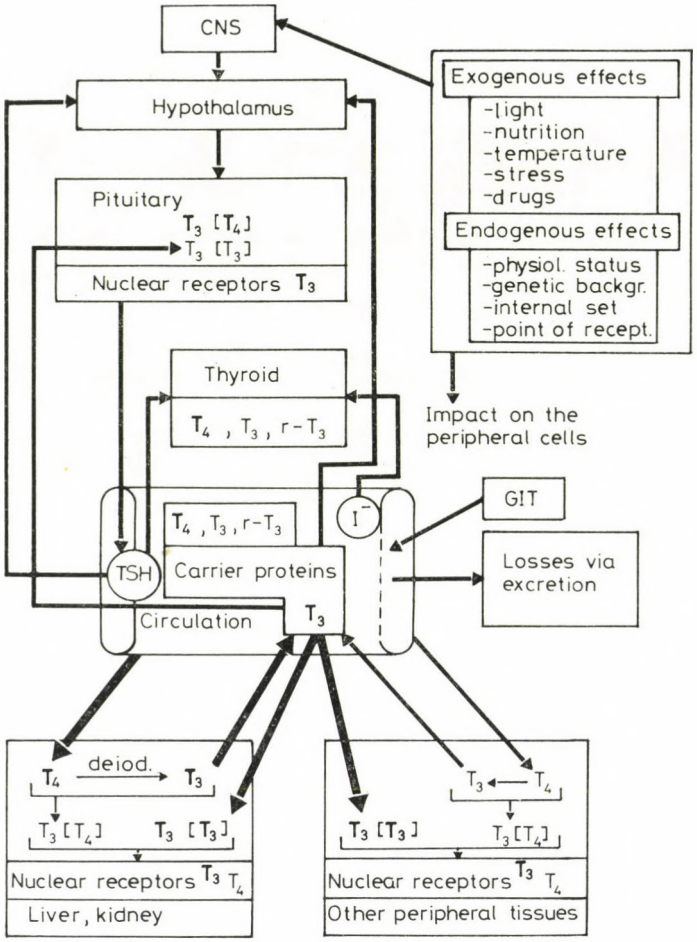

Fig. 1. Pathways of thyroid hormone action

signals, are depicted in Fig. 1 (for review see Larsen et al., 1981; Engler and Burger, 1984).

Environmental temperature, humidity, light cycle and climate are major factors altering CNS. This alteration is translated to TRH production or inhibition thereof at the hypophyseal level (Marshall et al., 1981; Péczely and Kiss, 1988). TRH at the same time was shown to have independent effect on the monodeiodinative ability of the liver in certain situations (Kühn et al., 1986), as a direct effect on the mobilization of the peripheral tissues. The other way of influencing thyroid from the outside is via the gastrointestinal tract: feed ingestion or the lack of it (energy content, protein/carbohydrate/fat ratio of the food, iodine content) can influence either thyroid function *per se*, or the metabolism of the individual organs (especially that of the liver), thus leading to altered activation of T4 (Balsam et al., 1981). The latter is pre-

sumably mediated by the gastrointestinal peptides, preceding the signals generated by the absorbed food itself (see later).

The HP will react on TRH action according to its intimately adjusted responsiveness predefined by the circulating amount of thyroxine, and also by its genetically defined ability to produce thyrotropin (TSH). TSH enhances all the known functions of the thyroid gland, upon which an increased amount of T4 and other iodocompounds will be secreted into the circulation. The level and binding characteristics of the binding proteins (BP) under genetic influence will modulate, but not basically determine, the availability of hormones for the cells (Hooman, 1981). According to the type of tissue, the hormones entering the cells will be activated, inactivated and redirected to the plasma pool or used for initiating cellular events characteristic of thyroid action. A considerable part (80%) of the circulating active thyroid hormone (triiodothyronine, T3) is coming from thyroxine converting tissues, and not from the thyroid itself. This led to the basically new concept that the amount of non-thyroidal T3 in different thyroid statuses is dependent on the metabolic state of tissues like liver, kidney, brain, etc. (Larsen et al., 1981; Silva et al., 1984). In the focus of this system are the deiodinating enzymes. This enzyme complex is responsible for generating active T3 from T4 entering the cell and is capable of successively deiodinating iodothyronines from T4 to T0, the thyronine skeleton. The research efforts on this field have revealed that the deiodinases can be classified into three groups (see Table I for an overview).

Type I enzymes deiodinate in both (the outer phenolic and the inner tyrosyl) rings. This enzyme is inhibited by 1 mM propylthiouracyl (PTU), a compound used to differentiate this type from the type II enzyme. The latter can only deiodinate iodine atoms at the 5'-position. The third type of enzyme (type III) is specific to the tyrosyl ring and deiodinates the 5-position iodides. It should be noted, however, that this classification cannot explain

Table I

Characteristics of iodothyronine deiodinases

	Type I	Type II	Type III
Organ specificity	liver, kidney, ubiquitous in other organs	central nervous system, hypophysis, brown adipose tissue	central nervous system, placenta
Sensitivity to propylthiouracyl	+	−	−
Site of action	both inner and outer ring	outer ring	inner ring
K_m for T4	1–2 μM	1.0 nM	50 nM
Dithiothreitol requirement	medium	high	low
Activity in hypothyroid states	decreased	increased	decreased

all deiodinative phenomena (Leonard et al., 1984). The exact nature of the enzyme proteins (or protein complexes) belonging to these groups has not yet been characterized. The type differences are of major physiological importance. The pituitary and the intrapituitary deiodination processes are basically important in elucidating some hitherto unclear phenomena. It is now obvious that the sensitivity of the pituitary to thyrotropin hormone (TRH) is not a function of the circulating amount of the active hormone, T3, but depends on the intrapituitary production of T4 through deiodination from T4 coming from the circulation (Silva and Larsen, 1982).

The active form of thyroid hormones (T3) thus available for the cells will exert the effects known as "thyroid hormone action". The most important way of these actions is that which activates the nuclear receptor thus leading to gene expression. Beside this basically important way there are other proved sites of action (mitochondrial, plasma membrane, enzyme activation and self-regulatory that are of interest (Dillmann, 1985).

Thyroid hormones (mainly T3) actively involved in the provocation of metabolic, catabolic and anabolic events are then released from their binding sites, deiodinated, conjugated or transaminated and excreted. During excretion, depending on the form of the excreted molecule, there are possibilities of reentry via the gastrointestinal tract, where well-defined microorganisms can release active thyroid hormones from their gluconate bonds. The final elimination (the "sink" in kinetic terms) occurs either via faecal or renal excretion. One can see from the above that the actual level of thyroid hormones in the blood, the apparent distribution volume, clearance rate, overall production rate and mean residence time of thyroid hormones especially with regard to individual organs or organ systems are under control of an overtly sophisticated mechanism. Simple measurement of thyroid hormone levels in blood, without monitoring at least some kinetic or deiodination parameters, is no longer feasible (for details of the above see Larsen et al., 1981; Wartofsky and Burman, 1982; Dillmann, 1985).

2. Examples underlying the new concept

2.1. Environmental temperature

The effect of environmental temperature is one of the factors known to be able to alter thyroid economy. It was shown earlier that cold environment is of stimulating, while warm is of depressing effect on the HT–HP–THY axis (Marshall et al., 1981; Wartofsky and Burman, 1982; Péczely and Kiss, 1988). Conflicting data were published, however, concerning the role of TSH elevation in this respect. Fisher and Odell (1971) could not show any increase of plasma TSH in humans exposed to cold. Also, polar conditions enhanced

Table II

Plasma concentration (T4, nmol/L±SEM, N = 10) and metabolic clearance rate (MCR, L/day/kg bw±SEM, N = 10) of thyroxine after exposing 4-week-old chickens to mild cold (10 °C)

	10 °C		23 °C	
	2 h	7 h	2 h	7 h
T4	5.1±0.75*	6.7±0.95*	15.1±1.3	14.3±1.1
MCR	4.5±0.31*	4.2±0.22*	3.1±0.14	2.8±0.12

* $P < 0.01$ vs. results with animals kept at 23 °C

TSH secretion only after a longer lag period that was preceded by the elevation of basal metabolic rate. The possibility that thyroid hormone metabolism in the peripheral cells can adapt to the changes in environmental temperature was raised by several research groups. This laboratory found (Decuypère et al., 1981; Rudas and Pethes, 1981) that there is an unexpected decrease of thyroxine serum concentration shortly (2 h) after exposing animals to mild cold (10 °C) and demonstrated that this is due to an increase of the metabolic rate of this hormone (Table II), the latter being the result of enhanced deiodination of thyroxine to the active triiodothyronine. This phenomenon precedes the increase of thyroxine output by the thyroid gland. Later it was shown that behind this early adaptation phenomenon a considerable increase of type-I deiodination in the liver can be detected (Table III).

Since then it was shown in several laboratories that the adaptation to cold environment starts with an adrenergic activation of several deiodinase systems independently of thyroxine mobilization from the thyroid gland. Recently it was proved (Silva and Larsen, 1983, 1985) that in rodents the type-II deiodinase of the brown adipose tissue is activated within hours during cold adaptation.

2.2. Feeding

With the advent of precise serum thyroid hormone measurement techniques it became evident that the feeding pattern and the quality of food

Table III

The activity of 5'-monodeiodinase (5'-D-type-I, pmol T3/mg DNA/h±SEM, N = 7–10) in the liver of chickens exposed to mild cold (10 °C for 1 h)

	10 °C	23 °C
5'-D type-I	13.61±1.4*	22.83±2.83

* $P < 0.01$ vs. 23 °C

Table IV

Serum triiodothyronine (T3, nmol/L±SEM, N = 5), liver 5'-monodeiodinase activity (5'-D-type-I, pmol T3/100 mg liver protein/30 min+SEM, N = 5) and thyrotropin releasing hormone provocation test (TRH, % of change in plasma T4 30 min after 1 μg/kg bwt TRH iv. ±SEM, N = 5) in chickens fed 100% (control), 85% and 70% of daily energy requirement in an isoproteic perfeeding trial 12 h after ingesting low energy diet

	100%	85%	70%
T3	1.54±0.13	0.99±0.21	0.56±0.17*
5'-D-type-I	28.6±1.45	20.1±1.28	17.5±0.11*
TRH	156±28	110±18	35.2±15.2*

* $P < 0.05$ vs. 100%

have a basic effect on thyroid hormone economy (Balsam et al., 1981; Wartofsky and Burman, 1982; Decuypère and Kühn, 1984).

It has been shown that feed intake and thyroid economy interrelate in several ways both in mammals and in birds. This interaction is thought to consist of at least two phases, the first of which occurs immediately after changes in the amount or quality of food available and the other appears after a lag period of usually some days. In a series of experiments with animals fed different energy levels it was proved (Suda et al., 1978; Harris et al., 1979; Burger et al., 1980; Bartha et al., 1987) that the decreasing amount of energy equivalents controls the activity of liver deiodination, a phenomenon that occurs parallel with the known changes (Burman et al., 1983) in the HT–HP–thyroid axis. As Table IV shows, the decreasing ingestion of energy inactivates liver type-I 5'-deiodinase within some hours (Bartha et al., 1987). This leads to a decrease in thyroxine degradation (T4 to T3 conversion) and to blunted sensitivity of the pituitary to TSH. In recent experiments we have demonstrated that the quick response of peripheral thyroid hormone metabolism

Table V

Effect of glucagon (7.5 μg/kg bwt, iv.) and of refeeding on the 5'-deiodinase type I in liver homogenates of 4-week-old chickens fasted for 16 h (% 125-I-T3 produced from 125-I-T4/30 min/g fresh liver *in vitro*)

	90 min after glucagon	120 min after refeeding	Non-treated	
			non-fasted	16-h fast
$\bar{x}$	34.2*	22.1*	27.2	15.4
SEM	3.5	2.1	2.8	1.0
N	(8)	(8)	(8)	(8)

*$P < 0.05$ vs. non-treated, 16-h fast

to changes in feed availability might be mediated by gastrointestinal hormones, first of all by glucagon (Table V) (Rudas and Newcomer, 1987).

2.3. Thyroid status

Since Sterling et al. (1971) proved the early finding of Roche et al. (1956) that thyroxine can be converted to triiodothyronine in mammals, several research groups have delivered evidence about the self-regulatory role of thyroid hormones themselves upon the rate of this conversion. Thyroid hormone depletion leads to a situation where particular organs of the body change deiodination rate in different ways. Thyroidectomy initiates a complex defense mechanism against hypothyroidism: liver type-I deiodinase decreases its activity while deiodination in the brain is increased (Silva et al., 1983; Silva and Larsen, 1982; Rudas and Pethes, 1989). The overall result of this mechanism is that thyroid hormones will be available for a long time in the hypothyroid organism (Obregon et al., 1981; Silva and Matthews, 1984) and the brain will become less hypothyroid than other organs so that it can avoid fatal "athyreotic" status for a long period after thyroidectomy. Similar adaptation of the peripheral thyroid hormone metabolism was seen in thyroidectomized chickens: the active thyroid hormone (T3) content in the brain was almost half of the euthyroid level, while plasma and liver thyroid hormone concentrations were dramatically reduced shortly after thyroidectomy (Rudas and Pethes, 1989). In these states the amount of thyroid hormones available from the plasma pool or from the local production is autoregulative in nature (Koenig, 1986; Rudas, 1988).

2.4. Effect of exogenous substances

Foreign substances introduced to the organism can influence thyroid hormone economy at several levels. It is of special interest that a wide variety of physiological, pathological and genetic disorders as well as drugs influence the dynamics of thyroid hormone transportation and deiodination. Detailed description of these effects can be found in excellent recent reviews (Wenzel, 1981; Cavalieri and Pitt-Rivers, 1981; Kaplan, 1984). In a series of experiments on quails and chicks we have demonstrated (Table VI) that the elevated nitrate (nitrite) concentration of the drinking water not only influences the thyroidal iodine uptake as it could be postulated on the basis of former findings, but it can also finely adjust the thyroid hormone activating pathways (liver 5'-deiodination) (Pethes et al., 1987).

Probably there is an enhanced demand on available triiodothyronine in cells under even light nitrate load. In another attempt we have seen that toxic substances like trichothecene derivatives can have a direct effect on the peripheral handling of thyroid hormones beside their effect upon the central

Table VI

Serum thyroxine (T4, nmol/L±SEM, N ± 5), liver 5'-deiodination (5'-D-type-I, pmol T3/100 mg liver tissue/30 min±SEM, N ± 5) and thyrotropin releasing hormone provocation test (TRH, % of change in plasma T4 30 min after 1 μg/kg bwt TRH iv. ± SEM, N ± 5) in adult quails exposed to 300 ppm nitrate in the drinking water for two weeks

	Control	Nitrate-fed
T4	7.5±1.8	3.5±1.1*
5'-D-type-I	32.4±2.8	47.5±3.2*
TRH	187±32	432±67*

* $P < 0.05$ vs. control

(HT–HP) system. These facts call attention to the fact that some well-known and formerly described phenomena are worth of reinvestigating in the light of the new regulatory mechanisms described in Part 1.

3. Conclusion

On the basis of the above one can conclude that when investigating thyroid hormone economy one has to take into account a series of recently established regulatory pathways that might be the cause of changes observed. One concludes therefore that measuring serum levels of thyroid hormones alone would not give a satisfactory explanation for thyroid economy. It is suggested that the state of peripheral activation (deiodination rates) and the sensitivity of the central axis (via TRH provocation tests) should be considered simultaneously when thyroid status is investigated.

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CORRELATION BETWEEN THYROID HORMONE LEVEL AND BLOOD pH IN COWS AND IN THEIR OFFSPRING

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Thyroid hormone levels in the plasma and blood pH were compared in 38 newborn calves and their dams immediately at parturition, then 24 and 72 h thereafter. Both thyroid hormone levels and pH exhibit much higher variation in calves than in cows. Applying a linear model at different sampling times, a weak ($r = -0.112$ to -0.397) but significant ($P < 0.05$) negative correlation was found between thyroid hormone levels and blood pH in calves 24 and 72 h postpartum. At birth, when no correlation was found with linear approach, a polynomial regression model showed curvilinear relationship between thyroid hormone levels and blood pH in calves. Since these correlations could not be substantiated by comparing data of different sampling times, one can conclude that the possible relationship of thyroid hormone levels and blood pH is a complex one. Revealing the underlying mechanism of the above observations requires further study.

Keywords: Thyroxine, triiodothyronine, blood pH, calves, cows

The plasma levels of thyroxine and triiodothyronine are influenced by different extrinsic and intrinsic factors (Larsen et al., 1981). One of the most important factors is plasma binding proteins that carry thyroid hormones to target cells. These thyroid hormone binding proteins associate with thyroxine and/or triiodothyronine with varying affinities and capacities in different species (for review see Larsson et al., 1985). An important factor that affects binding characteristics is hydrogen ion concentration (Davison et al., 1978). It is well known from the literature (Eigenmann et al., 1981; Held et al., 1985) and also in a series of experiments we have demonstrated (Szenci, 1985; Szenci et al., 1988) that blood acid–base status is critical for survival of the newborn calf. It is also known that the level of thyroid hormones exhibits a typical pattern around parturition (Curtis and Abrams, 1977) and that thyroid hormone levels in the young are in relation to rapidly changing metabolite levels of he blood (Farkas, 1982). It was shown (Pethes et al., 1978) that thyroid hormone levels in the serum immediately after birth may predict some production traits (meat quality, body weight gain) of the growing animals. Therefore it is reasonable to study the interrelationships of different blood parameters at the early stage of development. With this in mind, it was

worth carrying out a study that looks for relationships of blood pH with thyroid hormone levels. The present paper describes our first trial to demonstrate such interrelationships in newborn calves and their dams immediately after birth.

Materials and methods

Animals

A field study was performed in a dairy herd in September 1987. Dry cows (Holstein-Friesian and Holstein-Friesian × Hungarian Fleckvieh F_1 and R_1) were confined to stanchion barns and received a mixed commercial silage and fodder ration along with alfalfa and grass hay. Delivery of 38 calves, all in normal anterior presentation, was uncomplicated. One to three attendants assisted with traction. Delivery was completed within 30 to 60 min after the appearance and rupture of membranes. During the trial newborn calves were naturally suckled by their own dams.

Sampling

Immediately after birth, blood samples from the jugular vein were collected anaerobically into heparinized plastic syringes and into heparinized collection tubes which were immediately sealed airtight with a rubber cap and stored on ice until analysis. Blood sampling was repeated 24 and 72 h after birth. Heparinized blood samples were centrifuged (1500 *g*, 15 min, 4 °C) and the separated plasma was stored at —20 °C until analysis.

Assays

The hydrogen ion concentration of the blood samples was determined with a Biological Microanalyzer (Radelkis, Budapest, Hungary) at 37 °C within 10 min after sampling.

The concentration of thyroid hormones was determined according to our formerly described RIA method (Pethes et al., 1978). The procedure is similar to the widely used thyroid hormone determinations by RIA with the important difference that species-specific incubation environment was ensured throughout in the case of both hormones. This involved the use of thyroid hormone free serum made of bovine plasma for all dilutions of standards and samples. Applying this method a considerable improvement of assay accuracy was achieved over a wide range of hormone concentrations. The intraassay variance was less than 7 per cent, and the interassay variance was lower than 10 per cent.

Statistical analysis

The effect of pH on the plasma concentration as a function of sampling time was substantiated by analysis of variance (ANOVA, statgraphics procedure for IBM PC, Stat. Software Corp., USA). Non-linear regression analysis was done by approximation with polynomial and multivariate programmes.

Results

Table I summarizes pH, T4 and T3 data for both the calves and the dams. Hydrogen ion concentrations and thyroid hormone levels show greater fluctuations in calves than in the respective mothers. The blood pH was lower (7.1–7.3) and thyroid hormone level was higher in the plasma of newborn calves than in the cows. In calves blood pH increased after parturition, thyroid hormone levels achieved a peak concentration at 24 h and a decline was characteristic later on. The corresponding parameters did not change over time in the case of cows except that there was a slight drop in T3 serum concentration.

Table I

Blood pH, thyroxine (T4) and triiodothyronine (T3) levels in calves and in their dams (nmol/l)

			Average	±SEM
Calves	1 h	pH	7.22	$\pm 0.012^{A}$
		T4	172.2	$\pm 11.1^{B}$
		T3	7.81	$\pm 0.84^{C}$
	24 h	pH	7.38	$\pm 0.007^{A}$
		T4	303.2	$\pm 9.76^{B, D}$
		T3	12.76	$\pm 0.81^{C, E}$
	72 h	pH	7.39	±0.006
		T4	204.48	$\pm 11.87^{D}$
		T3	9.53	$\pm 0.56^{E}$
Cows	1 h	pH	7.41	±0.009
		T4	41.72	±3.12
		T3	2.04	$\pm 0.10^{A}$
	24 h	pH	7.44	±0.007
		T4	45.42	±3.17
		T3	1.94	±0.088
	72 h	pH	7.43	±0.006
		T4	43.37	±2.67
		T3	1.76	$\pm 0.12^{A}$

Average ± standard error of mean; n = 38; significant difference exists between groups labelled with the same superscript. $^{A, B, C, D, E}P < 0.05$ between groups with same superscript

Table II

Summary of regression analyses on the relationship between blood pH and thyroid hormone level (number of pairs = 38)

Regression of pH with		Correlation coefficient
Calves		
At birth	T4	–0.063
	T3	–0.067
24 h	T4	–0.173*
	T3	–0.256*
72 h	T4	–0.397*
	T3	–0.112*
Cows		
At birth	T4	0.092
	T3	–0.071
24 h	T4	–0.053
	T3	0.017
72 h	T4	0.005
	T3	–0.071

* $P < 0.05$ (significance of the correlation coefficient)

Regression analysis of pH on thyroid hormone levels was made at the different time points of sampling. A summary of the regression statistics is given in Table II. No significant relationship could be detected in the case of cows, however in calves a significant correlation of blood pH with plasma

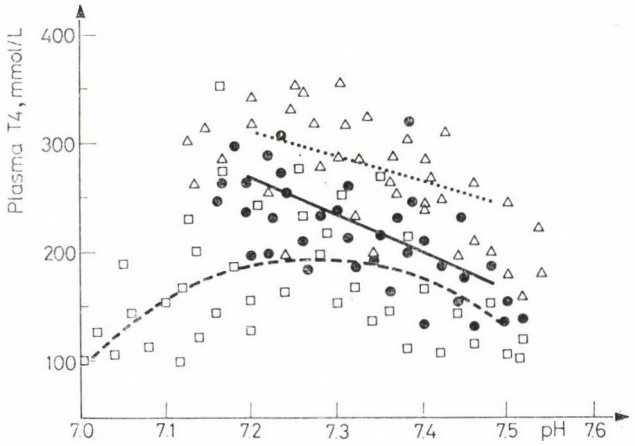

Fig. 1. Graphic representation of data on blood pH and T4 levels in calves. (□):at birth; (△): 24 h; and (●): 72 h postpartum. Overall inspection of data suggests no correlation. Detailed non-linear analysis within time points, however, resulted in a curvilinear relationship at birth (– – – –) and a close to linear negative correlation 24 (· · · · · ·) and 72 (———) h postpartum. The negative relationship of the two parameters is also apparent in the close to physiological (7.2–7.5) pH range at birth (□)

thyroid hormone level was found 24 and 72 h after birth. Figure 1 exhibits all the individual data of calves concerning T4. Visual inspection of all points together shows no correlation over the period investigated (i.e. 1, 24 and 72 h postpartum). However, when within-time-point data are looked at separately, one can reveal the following. Thyroxine level had a peak in the pH range of 7.20–7.30, while its concentration was lower at extremely high (7.30–7.35) and low (6.9–7.15) pH at birth. Thus non-linear polynomial regression was used to characterize this phenomenon. Figure 1 clearly shows the curvilinear relationship existing between T4 level and pH at birth. When a linear model was fitted to the full pH range at this sampling point, no correlation was found. An attempt was made to characterize this relationship by fitting a non-linear multiplicative model, the best fit of which was $y = ax^2$ (probability level is less than 0.05 per cent). When the more physiological pH range (7.2–7.5) is considered, the negative correlation still prevails (Fig. 1, open squares, pH range 7.2–7.5). At later time points (24 and 72 h after birth) a significant negative correlation of blood pH with thyroxine and triiodothyronine levels was confirmed with this approach (Fig. 1) as well as with the linear approach (Table II). Similar results were obtained for T3 with the above non-linear method (data not shown).

Discussion

The data presented above show that the blood parameters measured have very high variance in the case of calves but not in that of cows. In general, one can observe that blood pH is very low immediately after birth, and due to metabolic compensation of the acid–base balance its value returns to normal physiological levels (Eigenmann et al., 1981; Szenci, 1985). The blood pH values of the dams are in good agreement with those described in the literature as physiological (Szenci, 1985). The high level of thyroid hormones found in the calves is not surprising, knowing that thyrotropin (TSH) is suddenly released upon the cold environment and this increases T4 level (Fisher and Dussault, 1975; Pethes et al., 1985) and that peripheral deiodination of T4 to T3 is extremely high in the early postnatal period (Cavalieri, 1980) accounting for very high T3 levels. Absolute values of T4 and T3 in cows and the phenomenon that thyroid hormone levels of cows do not change significantly over the first 72 h postpartum agree with the literature (Fisher and Dussault, 1975; Pethes et al., 1985).

Detailed analysis of data suggest that there is a correlation of pH with thyroid hormone levels. Since all data of calves (including observations at birth) in the range of pH 7.25–7.5 negatively correlate with pH (Fig. 1), one can conclude that high hydrogen ion concentration increases binding of

thyroid hormones. Apparently the same is seen when data from cows are compared to those found in their offspring. Calves have high thyroid hormone levels and low pH, cows show low thyroid hormone levels and higher pH. However, this relationship seems not to be true when all data from calves are compared. Due to the numerous indirect effects, pH can only be one of the several metabolic factors that influence thyroid hormone level in such a comparison and the relative influence of these factors may change over time. The negative correlation found in calves is supported by results of Davison et al. (1978) who showed that characterization of thyroid hormone carrying proteins above the physiological pH resulted in severe underestimation of binding constants. When the very early data on calves (immediately after birth) are considered, one concludes that above a certain critical concentration (below pH 7.2) hydrogen ion might inhibit thyroid hormone binding by serum proteins.

Thyroid hormone level in the plasma is influenced by several factors (production, elimination, etc.), one of which can be the binding characteristics of hormone binding proteins. It would be an oversimplification, however, to state the weak relationship found above would express a direct pH–hormone level correlation. A very important factor that might also be included in the phenomena found here is the altered metabolite level of the calf serum around birth. Fatty acids, hormones, lactate and other compounds can directly replace thyroid hormones from their plasma binding sites (Tikanoja and Liewendahl, 1988; Lim et al., 1988; Schifferdecker et al., 1988) and this can indirectly lead to the observed correlation of hydrogen ion concentration with thyroid hormone levels at the very early time point after birth in calves. The first movements and muscle work can also alter the binding of thyroid hormones (Pakarinen et al., 1988) and this might be an obvious reason in the newborn calf. Also, it is well known that thyroid status itself alters serum binding of thyroid hormones, and that might be independent of pH (Leopold et al., 1988; Tanaka, 1988; Young et al., 1988). The data presented here support the view that pH may correlate with thyroid hormone levels at certain time points. The diversity of possible underlying mechanisms (Davison et al., 1978; Benvenga et al., 1988; Terasaki and Pardridge, 1988; Shigemasa et al., 1988) and the inconsistencies observed require more detailed analysis of the above findings.

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THE EFFECT OF TANNIN TREATMENT AND SUBSEQUENT UREA SUPPLEMENTATION OF SUNFLOWER MEAL ON THE *IN VITRO* DIGESTIBILITY OF ITS CRUDE PROTEIN FOR RUMINANTS

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The effect of tannin treatment (TSFM) and subsequent urea supplementation (TSFM + U) on the digestibility of sunflower meal (SFM) protein was studied by means of a three-phase laboratory method simulating the ruminant's digestion. Under the influence of the ruminal fluid 67% of SFM protein, 62% of TSFM protein and 58% of TSFM + U protein was degraded. The pepsin and pancreatin digestibilities of rumen undegraded protein (UDP) were as follow: 58.2% (SFM), 56.6% (TSFM), 43.3% (TSFM + U), and 22.0% (SFM), 28.8% (TSFM), 17.5% (TSFM + U), respectively.

The four fractions (dissolved protein, oligopeptides, amino acids, and ammonia) of rumen degradable protein (DP) were also determined: 85 to 92% of rumen degradable protein was recovered in these four fractions. Owing to tannic acid treatment the quantity of dissolved protein decreased and that of oligopeptides increased.

Keywords: *In vitro* protein degradability in rumen, digestibility of undegraded protein, extracted sunflower meal, tannin treatment, urea supplementation, digestion of crude protein, crude protein fractions, rumen degraded protein

Some plants (*Lotus pedunculatus, L. corniculatus* and some bird-resistant *Sorghum* types), widely used in animal nutrition, contain different amounts of condensed tannins (Barry et al., 1986; Barry and Forss, 1983; Waghorn et al., 1987; Montgomery et al., 1986). During disintegration of the plant material, such as chewing by ruminant, the condensed tannins react with plant proteins to form an insoluble complex. The latter is stable and insoluble in the pH range of 3.5–7.0, but will dissociate at pH below 3.5 (Jones and Mangan, 1977). Therefore, proteins in plants containing tannin can be protected from microbial degradation in the rumen (Waghorn, 1985).

It was shown (Sengar and Mudgal, 1982) that the solubility of groundnut cake protein was reduced by tannic acid treatment. The changes in solubility depended upon the amount of tannic acid used. The objectives of the present investigation were to determine the effect of tannin treatment of sunflower meal on the *in vitro* digestibility of its protein. The effect of a subsequent urea supplementation on the digestibility of the treated protein was also studied. The quantities of the four fractions of rumen degradable proteins (DP) were measured too.

Materials and methods

Commercial SFM (crude protein content: 424 g/kg) served as untreated feed. The production procedure began with the mechanical dehulling of the sunflower seed. After having heated the dehulled seeds up to 70–80 °C, the oil was removed by pressing and subsequent chemical extraction at 40 °C. The solvent residue was eliminated by toasting, then the extracted SFM was ground. (During the described process, for a short time the temperature reached 100 °C.)

Tannin treatment was carried out using 21% tannic acid solution. Sunflower meal was laid out uniformly in a layer thickness of 5 cm and was sprinkled with the tannic acid solution. The quantity of tannic acid used was 9% of the crude protein amount of SFM. The humid feed was dried by ventillation for 48 h to air-dry state.

Rumen fluid was taken from 3 adult, fistulated Merino wethers. The animals were fed mixtures containing SFM, TSFM or TSFM + U (Table I), following a Latin square design. After the third treatment period all animals were fed a control diet. Each sampling and determination was preceded by a 10-day adjustment period during which the animals consumed a feed containing the same sunflower meal, whose degradability and digestibility were

Table I

Composition and nutritive value of the daily ration of sheep

Ingredients	Group		
	I	II	III
Ground corn grain (kg)	1.07	1.07	1.07
SFM* (kg)	0.25	—	—
TSFM* (kg)	—	0.25	0.25
Alfalfa hay (kg)	0.20	0.20	0.20
Wheat straw (kg)	0.10	0.10	0.10
Mineral (g)	16.00	16.00	16.00
Urea (g)	—	—	9.00
Specifications:			
Dry matter intake (kg)	1.46	1.45	1.53
Digestible energy intake (MJ)	20.94	20.72	20.72
Crude protein intake (g)	219	206	248
Ca intake (g)	4.1	4.1	4.1
P intake (g)	3.3	3.3	3.3

* SFM: sunflower meal, extracted; ** TSFM: tannin-treated sunflower meal

determined *in vitro*, by means of their rumen fluid. The composition of the three feed mixtures is shown in Table I.

The *in vitro* measurements were made by a three-phase method (Veresegyházy et al., 1987; Veresegyházy et al., 1989). In the first phase 5×2 incubations were done with the rumen fluid of each animal, using 500 mg test material per incubation. Urea was dissolved in McDougall's buffer (1948), in a concentration of 0.125 (w/v) %. Each measurement series included a blank test (incubation of the rumen fluid without feed sample). At the end of the 48-h incubation period, digestion was stopped by adding 5% $HgCl_2$ solution, and the tubes were centrifuged (8000 *g*). From half of the samples (i.e. 5 parallels) the supernatant was discarded, while the sediments were further digested in the second phase with pepsin and in the third one with pancreatin. The sediments of the other half of the samples (5 parallels) were used for the determination of the amount of rumen undegraded protein (UDP). Their supernatants served for measuring the crude protein fractions of rumen degradable protein (DP).

For UDP determination, nitrogen content was measured in the sediments of the first phase. For the determination of the pepsin and pancreatin digestibility of UDP, the nitrogen concentration of the supernatants of the second and third phases was measured. Determinations were performed by the Kjeldahl's method, using a Kjell-Foss instrument. Crude protein was obtained by multiplying the result by 6.25. For the lack of homogeneity the entire quantity of the sediments was used up.

The crude protein fractions of the DP were determined as follows. Using 1 ml of the first-phase supernatants, the biuret test (Rapoport and Raderecht, 1984) was performed to estimate the total quantity of dissolved peptides and proteins. Bovine serum albumin (BSA) dissolved in 0.9% NaCl at different concentrations was used as standard solution.

2.5 ml of the first-phase supernatant was deproteinized by adding the same volume of 10% trichloroacetic acid. After centrifugation the supernatant was used for the biuret test, Sörensen's formol titration (Rapoport and Raderecht, 1984) and ammonia determination by Berthelot's method (Keller et al., 1967). Oligopeptide concentration was measured by biuret test of the deproteinized supernatant. The quantity of dissolved protein was calculated from the difference between the intact and the deproteinized supernatant. The concentration of free amino acids was obtained by multiplying by 120 (i.e. the mean molecular weight of amino acids) the numbers of NaOH moles used up during the formol titration. Ammonia quantity was determined in 0.2 ml of deproteinized supernatant from the first phase by Berthelot's method. The intensity of the blue colour formed was measured by photometry at a wavelength of 550 nm. A 1% $(NH_4)_2SO_4$ solution was used as standard.

Results

During the experiment each animal consumed a tannin-containing feed mixture for 20 days (10 days TSFM+10 days TSFM+U). Voluntary feed intake did not change, but the animals excreted a less consistent, cattle-like faeces.

Ruminal degradability of the crude protein (CP) was calculated as the difference between the protein content of the initial material and the sediment of the first phase. Given the fact that the intact and treated sunflower meal differed in crude protein concentration, the results are referred to 1000 *g* of CP. The measured individual UDP values are shown in Table II.

The average DP and UDP values of the three treatments (Table III) differed significantly. Under the influence of the rumen fluid the degradation ratio of SFM crude protein was 67%, that of TSFM was 62%, and that of TSFM+U was 58%. The differences between SFM and TSFM were significant

Table II

Changes in UDP content of crude protein after different treatments

Sample / Animal	SFM 1st measurement	SFM 2nd measurement	TSFM	TSFM+U
	g UDP/1000 g crude protein			
1	417	300	442	280
	322	210	332	465
	295	223	365	501
	293	319	535	515
	308	380	318	342
Mean	327	286	398	421
2	338	367	259	295
	391	302	521	500
	361	321	281	509
	385	342	378	292
	350	358	382	453
Mean	365	338	364	410
3	329	278	356	351
	346	387	436	493
	300	278	327	408
	353	364	419	392
	338	278	315	553
Mean	333	317	371	440
	n=30	n=15	n=15	
Mean of all data	328	378	423	
±SEM	8.7	14.7	16.9	

Table III

In vitro digestibility of crude protein of SFM, TSFM and TSFM + U

Crude protein fraction	Item	Animals 1	2	3	n	Mean	±SEM	Significance (P <)
		g protein/1000 g crude protein						
DP	SFM	694	649	675	30	672	8.7	—
	TSFM	602	636	629	15	622	14.7	0.01
	TSFM+U	579	590	560	15	576	16.8	0.001
UDP	SFM	307	352	325	30	328	8.7	—
	TSFM	398	364	371	15	378	14.7	0.01
	TSFM+U	421	410	440	15	423	16.8	0.001
digestibility by pepsin	SFM	147	201	224	30	191	10.1	—
	TSFM	174	255	214	15	214	14.0	ns
	TSFM+U	268	147	134	15	183	17.0	ns
digestibility by pancreatin	SFM	53	110	53	30	72	10.2	—
	TSFM	113	88	126	15	109	8.5	0.05
	TSFM+U	55	36	131	15	74	9.5	ns
total digestible UDP	SFM	200	311	277	30	263	15.0	—
	TSFM	287	343	340	15	323	17.4	0.05
	TSFM+U	323	183	265	15	257	17.9	ns
Total digestible crude protein	SFM	894	960	952	30	935	15.9	—
	TSFM	889	979	969	15	946	19.7	ns
	TSFM+U	902	773	825	15	833	18.3	0.01

ns = not significant;
SFM = untreated extr. sunflower meal;
TSFM = tannin-treated extr. sunflower meal;
TSFM+U = tannin-treated extr. sunflower meal with urea supplementation

on a level of $P < 0.01$ and those between SFM and TSFM+U were significant on a level of $P < 0.001$.

The *pepsin digestibility* of UDP (43.3–58.2%) was practically not influenced either by tannin or by subsequent urea supplementation.

The *digestibility of UDP by pancreatin* was nearly the same for SFM and TSFM+U (17.5 and 22.0%), whereas the protein digestibility of TSFM UDP was significantly ($P < 0.05$) better than that in the other two groups.

The total digestible part of UDP was the highest for TSFM (32.3% of the CP). The values for the other two groups were very similar (26.3 vs. 25.7%). The difference of the TSFM group from the two others is significant ($P < 0.05$).

The total "digestible" crude protein amount (DP+pepsin and pancreatin digested quantity) was not influenced by tannic acid treatment. Urea supplementation considerably decreased the overall *in vitro* digestibility (by 12%).

Eighty-five to 92% of DP was recovered in the above-mentioned four crude protein fractions (Table IV). The quantity of ammonia slightly decreased owing to the treatments (158, 127 and 124 mg/g CP, respectively). Changes

Table IV

Crude protein fractions of DP after *in vitro* incubation

Crude protein fraction of DP	Item	Animals 1	2	3	n	Mean	±SEM	P <
Dissolved proteins (g/1000 g CP)	SFM	170	94	52	30	105	11.8	—
	TSFM	35	113	0	15	49	10.1	0.01
	TSFM+U	21	51	11	15	28	6.2	0.001
Dissolved oligopeptides (g/1000 g CP)	SFM	40	36	80	30	52	7.3	—
	TSFM	123	26	103	15	84	8.4	0.05
	TSFM+U	96	92	108	15	99	4.9	0.001
Free amino acid (μmol×120 g/1000 g CP)	SFM	255	307	306	30	289	14.9	—
	TSFM	287	302	351	15	313	11.7	ns
	TSFM+U	264	209	242	15	239	23.6	ns
Ammonia-N×6.25 (g/1000 g CP)	SFM	156	186	133	30	158	11.3	—
	TSFM	205	92	85	15	127	10.6	ns
	TSFM+U	128	135	110	15	124	6.2	ns
Sum of different DP fractions (g/1000 g CP)	SFM	621	625	573	30	604		
	TSFM	634	531	528	15	573		
	TSFM+U	498	474	459	15	490		
Percentage of DP	SFM	89	93	85	30	90		
	TSFM	105	84	84	15	92		
	TSFM+U	86	80	82	15	85		

SFM = untreated extr. sunflower meal; TSFM+U = tannin-treateg extr. sunflower meal with urea supplementation
ns = not significant;
TSFM = tannin treated extr. sunflower mear;

in the amount of free amino acids were within the range of the standard deviation. The increase in the quantity of dissolved oligopeptides was significant ($P < 0.05$ for the TSFM and $P < 0.001$ for the TSFM+U treatment), as well as the decrease in the amount of dissolved protein ($P < 0.01$ and $P < 0.001$, respectively).

Discussion

The rather high (33–44%) protein content of extracted sunflower meal (SFM) has a very favourable amino acid composition. Its utilization in ruminant nutrition is hindered by the fact that a significant proportion (65–75%) of its protein will be broken down and partly built into microbial protein. During this process part of the protein of high biological value suffers transformation losses, the formed microbial protein has lower biological value and only the remaining relatively small proportion will serve directly for the amino acid supply of the host animal. Any kind of physical or chemical treatment, which decreases the ruminal degradability of SFM, will improve the supply of high-producing cows with good-quality proteins, which are digestible in the small intestine.

According to data of the literature the protein solubility and *in vitro* digestibility of some condensed tannin-containing plants are in inverse ratio to their tannic acid content (Barry and Forss, 1973; Montgomery et al., 1986). Knowing that exogenous tannin decreased also the solubility of feed proteins (Sengar and Mudgal, 1982), we supposed that the ruminal degradation could be diminished in some way.

Although there is a series of data indicating that ruminal protein degradation and urea utilization are considerably influenced by the feeding level (ARC, 1984) and protein content of the daily ration (Voigt et al., 1983), because of its simplicity, repeatability and standardizable conditions we chose the three-phase *in vitro* method. This latter enabled us to determine the composition of the DP fraction too. As the ruminal degradability of proteins depends mostly upon the peptide uptake of the microbes (Chen et al., 1987), it seemed important to know the quantity of the arising oligopeptide.

According to the laboratory measurements tannic acid treatment significantly increased the UDP content and, consequently, decreased the proportion of DP. Since, as compared to the untreated control, the pepsin digestibility of this UDP did not change and its pancreatin digestibility improved, the total quantity of sunflower protein available in the intestine increased too. This rise was nearly 23% and was statistically significant. The sum of degraded crude protein during the three phases was practically the same in the case of SFM and TSFM (90 vs. 92%); only the proportions of degradation due to different effects changed. (Namely, during the procedures aimed at improving the bypass value of feed proteins, the danger exists that not only ruminal degradability but also intestinal digestibility decreases as a result of extreme protein denaturation).

Our *in vitro* method made possible the further investigation of the rumen degradable protein fractions. It can be stated that the decomposition of DP is not complete, so a part of the measured degraded protein (dissolved proteins, oligopeptides and free amino acids) can also contribute to the amino acid supply of the host animal. Among these fractions Chen et al. (1987) emphasize the importance of oligopeptides, declaring that the rate of ruminal deamination is regulated by the peptide uptake of the bacteria.

Tannic acid treatment did not influence the total quantity of DP fractions containing amino groups (446 mg/g CP in both groups). There was only a shift of proportions in favour of oligopeptides and amino acids. The intestinal digestion of these two fractions is better and quicker than that of untreated (intact) extracted sunflower meal. Consequently, it probably results in better protein utilization. The decrease in NH_3 concentration was not significant.

Urea supplementation caused a further 12% increase in UDP content. This was expectable, as urea is a more easily available nitrogen source for

microbial protein synthesis than is the feed protein. In the case of this latter treatment the pepsin and pancreatin digestibility of UDP was practically the same as that of the control SFM. The *in vitro* utilization of crude protein during the three phases (i.e. the sum of DP and total digestible UDP) was lower than in the other two treatments. We conclude that this phenomenon may be due to the following: increased microbial protein resulting from urea supplementation offers too large substrate supply for pepsin and pancreatin. The enzymes first split the microbial protein, because the UDP fraction of the feed is less digestible. This will not be reflected in the measurements because the blank tubes also contain urea. Our supposition is supported by the CP difference between the urea-free and urea-containing blank tubes (49 vs. 88.5 mg, respectively).

The ratio of the sum of the three DP fractions containing α-amino group is practically the same as in the other two treatments (TSFM+U: 75; SFM: 74; and TSFM: 78% of the DP). The ratio of dissolved oligopeptides and proteins is the highest in the case of TSFM+U treatment (3.5 vs. 1.7 for TSFM and 0.5 for SFM).

Ruminal protein degradation is probably more efficient under *in vivo* circumstances. Namely, the end products, which inhibit microbial metabolism, can leave the rumen during digestion. On the contrary, ammonia, accumulating in the incubation mixture, can hinder the activity of microbial deaminases, thus increasing the fractions that contain α-amino group.

Owing to the increase in the amount of UDP, tannic acid treatment seems to be suitable for partial protection of SFM protein from microbial degradation and consequently, for increasing the quantity of protein reaching the intestine. In the ruminant, the effect measured *in vitro* is probably complemented by enhancement of the outflow rate, which causes a further increase in the UDP fraction.

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INTESTINAL LYMPH OF RUMINANTS I. OPERATIVE TECHNIQUES FOR COLLECTING INTESTINAL LYMPH FROM RUMINANTS

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Operative techniques developed for collecting intestinal lymph from cattle and sheep are described. The method of creating an external anastomosis between intestinal lymph and the posterior caval vein (lymph–caval anastomosis), the postoperative care of animals, and the blood sampling technique are presented in detail. The optimal period of use of the anastomosis is 15 to 18 days. Its further use is of no reason, since the intestinal lymph changes in quality.

Keywords: Intestinal lymph, ruminant, operative techniques, lymph collection, anastomosis

The lymph circulation of ruminants has not been investigated in detail. The works available are mostly concerned with the thoracic lymph duct.

Our first study on the intestinal lymph included catheterization of the intestinal lymph duct with catheters that were exteriorized. Lymph was collected in polyethylene sacs, similarly to the technique described by Lascelles and Morris (1966) for sheep. The obtained lymph with heparin admixture was then permanently reinfused, aseptically, into the rumen or the jugular vein. The 12 sheep used in our experiment remained in a good physiological condition, and during the 8 to 12 days of lymph collection they lost no liveweight. However, in similar experiments, three young bulls became weak soon after surgery; we failed to maintain these animals in a normal state, therefore, lymph collection from them was ended.

Later, in 3 sheep and 2 young bulls, we attempted anastomosing the intestinal lymphatic trunk catheter to the mesenteric vein. Normally, a significant lipid amount is transported in the lymph into the systemic circulation, bypassing the liver. We tried to change this pathway, i.e. to make the lymph (with the lipids in it) enter the liver. Under such conditions, the physiological condition of the animals soon became worse, and lymph clotting in catheters occurred on the third to fifth day after surgery. In this experiment, we were especially interested in liver functions, in particular the exocrine function. Therefore, we carried out in three young bulls simultaneous cannulation of both the intestinal lymphatic trunk and the bile duct (Aliev and Alieva, 1966).

The connection of the lymphatic trunk with the mesenteric catheter resulted in a depression of bile secretion, thus reflecting a negative effect of the lymph upon liver function when a direct lymph influx into the liver occurred. Anastomosis between the intestinal lymphatic trunk and jugular vein catheters in 2 sheep did not give positive results, due to the extreme mobility of the animals' neck and the long distance between location sites of the lymphatic (near the spine) and venous (on the neck) catheters.

Earlier, we developed the "lymph–lymphatic" (intestinal trunk–intestinal trunk) anastomosis. The trunk was cut through between two ligatures, the first catheter was directed towards the pancreas (against the lymph flow), the second one was directed towards the cisterna chyli (with the lymph flow). Being properly fixed, both catheters were exteriorized and anastomosed on the abdominal wall. Lymph collection succeeded only in 4 of 10 yearling bulls over the first three to five days after surgery. In the other 6 animals lymph clotting occurred, possibly due to the absence of suckling effect in the system.

External connection of the intestinal lymphatic trunk with the caudal vena cava appeared to be physiological, and it was easy to perform. In this case, the natural pathway of the lymph flow did not change, although the lymph moved through the caudal vena cava rather than the cranial one. In both cases it flew through the right atrium into the systemic circulation. Moreover, such an approach proved to be more effective, since the catheter of the caudal vena cava, with the clamp put on it, made simultaneous sampling of efferent blood possible (Aliev, 1969).

Lymph–caval anastomosis

Anatomy. The topography of the intestinal lymphatic trunk of cattle has not been described in the literature. We have found that the trunk arises from the dorsal edge of the pancreas parenchyma, anterior to the right kidney. It runs caudally along the ventrolateral surface of the caudal vena cava and passes through the venous angle formed by the caudal vena cava and the left renal vein (Fig. 1), entering the cisterna chyli, which is found on the medioventral surface of the caudal vena cava and the lateral surface of the abdominal aorta. At the site of its transition from the dorsal edge of the pancreas parenchyma to the ventrolateral surface of the caudal vena cava, the trunk is joined by the hepatic duct. The diameter of the intestinal lymphatic trunk varies with age, ranging from 1.5 to 3.0 mm in sheep and from 2 to 8 mm in cattle. In young cattle, as a rule, the gastric lymph duct is connected with the cisterna chyli by an independent trunk. This is at variance with the widely-accepted opinion suggesting that the intestinal lymphatic trunk is a collector of the total lymph efferent from the complex stomach and the intestine, a view partly outlined by Lascelles and Morris (1966) in the descrip-

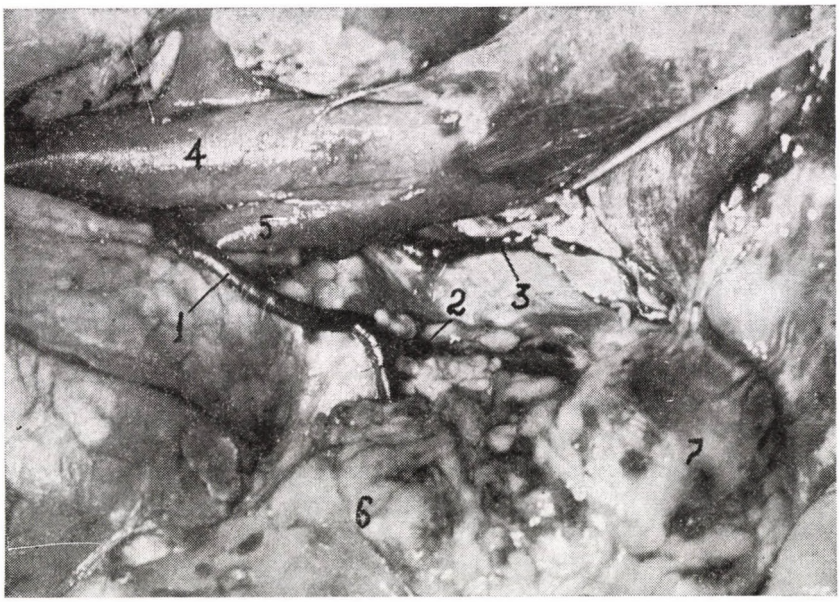

Fig. 1. Topography of lymphatic ducts. The intestinal (1), hepatic (2) and general gastric (3) ducts. The caudal vena cava (4), left renal vein (5), the pancreas (6) and the hepatic lymph node (7) are exposed

tion of the intestinal lymphatic trunk of sheep. Our studies in sheep revealed a significant variability of the intestinal and gastric lymph ducts (Fig. 2). As shown by duct preparation in 17 sheep, the gastric duct is connected in 88% of animals independently of the cisterna chyli.

Experimental animals. Clinically healthy, well-developed, full-blooded animals in a medium body condition were chosen for the study; lean and fat animals were excluded. One- to two-year-old sheep and young cattle tolerated the surgery well. Priority was given to ewe-lambs and heifers over male animals. The selected animals had good appetite and showed a calm behaviour.

Surgery. Over the period 1963 through 1981 we carried out surgical preparation of 55, 1.5- to 2-year-old ewe lambs, 127, one- to two-year-old heifers and 32, 1- to 1.5-year-old bulls. The percentage of unsuccessful operations, due to an indistinct topography of the lymphatic ducts, was 12 in sheep and 8.3 in young cattle. The operated sheep and cattle were used in experiments for 12 ± 3 and 15 ± 4 days, respectively, after surgery; the animals remained in a good physiological condition and the lymph–caval anastomosis functioned well. In one heifer the anastomosis remained patent for 40 days.

Fixation of animals. The animals which had been starving for 24 h were placed in left lateral recumbency, and firmly fixed to the operating table.

Anaesthesia. For general anaesthesia, 33% alcohol in saline (35 g per 100 kg liveweight) was infused into the jugular vein. For local anaesthesia,

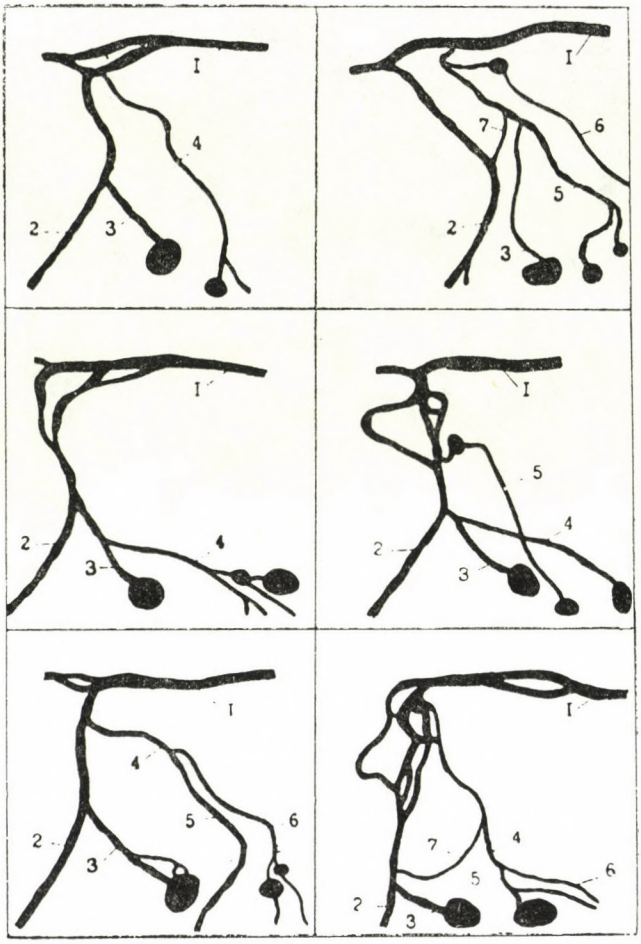

Fig. 2. Variants of junctions of lymphatic ducts of the stomach, intestine and liver. On each scheme designated are: the thoracic (1), intestinal (2), hepatic (3), general gastric (4) and ruminal (5) ducts, the general duct of reticulum, omasum and abomasum (6) and the anastomosis (7)

6% novocaine dissolved in saline (20 ml injection) was injected to the origin of spinal nerves, between the tenth and eleventh, and the eleventh and twelfth ribs, then 2% novocaine solution was infiltrated along the line of the incision to be made.

An incision about 35 to 40 cm long was made at the mid-portion of the thirteenth rib. The skin and the underlying tissues were dissected. In the upper end of the wound, as close to the spine as possible, the rib was transected by a small wire hand-saw and was removed. Then the abdominal cavity was opened by successive dissection of the periosteum, fascia and peritoneum.

Placement of gas-removing tube in the rumen. After laparotomy, a gas-removing tube, 1.5 to 2 m long, was implanted into the dorsal ruminal sac to prevent the rumen from dilating, an event disturbing the operation. The dilated rumen may limit the access to the other organs lying in the abdominal cavity, even in fasting animals, thus producing the risk of asphyxia.

As seen after the laparotomy, the rumen usually protruded on its right side, between the abomasum and the intestine, making the latter draw back and down. The rumen wall was distinctly seen under the external major omental lamina. A small cut was made in the epiploon by a closed method, through which the rumen wall was gripped and it was isolated with an antiseptic cloth. A purse-string suture was made in the rumen wall, and in the middle of the suture a small cut was made with a pointed scalpel for insertion of the flanged end of the polyethylene tube. The suture was then tightened and tied by a "surgical knot", the second move of the thread being left in the form of a loop. The suture was treated with streptocide, a small napkin was fastened by a ligature to the entry site of the tube into the rumen, and the whole construction was pulled down within the abdominal cavity. Gases left the rumen through the implanted tube; the markedly improving condition of the animal facilitated further procedures. The gas-removing tube was withdrawn at the very end of the operation, i.e. when 2/3 of the wound was already closed. For this purpose, the implant site for the tubes was drawn up in the wound, the napkin was removed, the loop undone and the purse-string suture loosened. After withdrawing the tube, the suture was tightened again with simultaneous inversion of the margins of the rumen mucosa. This area was treated with iodine, and antibiotics were applied. The aperture made in the omental lamina was also sutured.

Catheterization of the intestinal lymphatic trunk and caudal vena cava. A set of catheters used for the formation of the lymph–caval anastomosis is demonstrated in Fig. 3. After the placement of the gas-removing tube, the assistant retracted the exposed portion of the intestine downward and backward, with the help of a napkin made of enbleached calico, moistened with saline. Deep in the wound, the pancreas was found with the caudal liver process overflown upon it in the form of a sail. The separation of this fold by a closed method exposes, beneath the spine, the caudal vena cava, being adjacent to the dorsal edge of the pancreas. (The operator should give particular care to this step, since the liver can easily detach from the caudal vena cava or can be injured by a consequent haemorrhage. The blood soon fills the area of the trunk location, thus complicating the procedure.)

The intestinal lymphatic trunk was identified caudal to the liver on the ventrolateral surface of the caudal vena cava, in the fascia of the vein, as a white ligament disappearing in caudal direction, between the left renal vein and the caudal vena cava. After the preparation of the trunk, the caudal vena

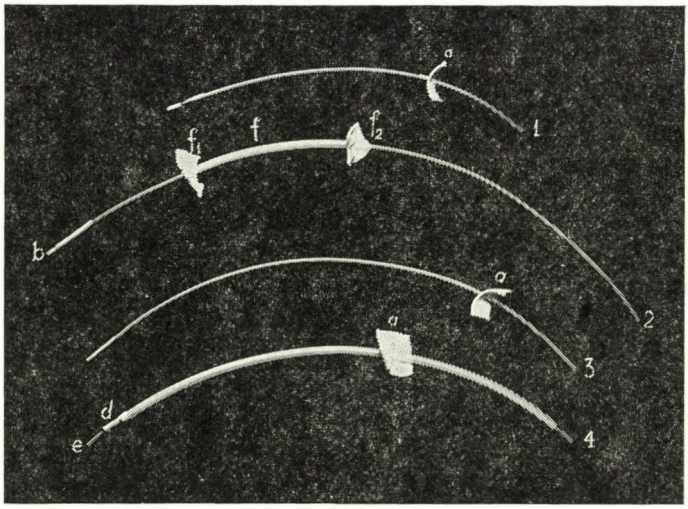

Fig. 3. A set of catheters: the arterial (1), portal (2), lymphatic (3) and caval (4). Catheters 1, 2 and 3 have the lavsan collar (a), serving as an anchoring device, and the plug (b). The rubber tubing on catheters 1 and 2 serves as a pathway for the syringe needle. Catheter 4 has the coupling (d) which seals the lumen of the catheter and through which the sampling incatheter (e) is inserted. The cannula (f), fixed on catheter 2, is stabilized by two lavsan collars (f_1 and f_2) to the neighbouring tissues, at the implant site of the catheter, and to the skin at the site of catheter exteriorization, thus stabilizing catheter 2 within the portal vein

cava and the orifice of the right renal vein, the actual procedure consisted of two stages, viz. catheterization of the intestinal lymphatic trunk, and that of the caudal vena cava.

Taking into account the topographical and surgical situations, we have developed several techniques for catheterization of the intestinal lymphatic trunk.

Technique 1. The trunk is cut across at the venous angle between two ligatures. The ends of the ligature, placed close to the cisterna chyli, are cut, those of the second ligature are conserved and fixed to the incision margins in the upper corner of the wound. Then a third ligature is slipped under the trunk, at the site of its transition from the pancreas parenchyma to the caudal vena cava, anterior to the entry of the hepatic lymph duct into the vein, and the long ends of this ligature are fixed to the incision margins. The thread should be loose enough not to strain the trunk. The latter fills with lymph well enough and becomes easy for catheter insertion. Pulling the ends of the second ligature, the operator elevates the prepared portion of the trunk and puts the index finger of his left hand beneath it. Then, by fine straight scissors, a partial cut in the trunk wall is made, while the assistant slightly tightens the third ligature at the orifice of the trunk from the pancreas parenchyma, thus preventing lymph leakage. Immediately, the operator introduces the catheter tip into the lumen of the trunk by means of a stylet. The second

ligature is loosened, then tightened again, and tied when the catheter tip advances for approximately 2.0 cm within the pancreatic portion of the trunk. One of the ends of the second ligature is wound around the catheter, above the lavsan collar, and is tied together with the other end of the same ligature. A fourth and, if necessary, a fifth ligature is made between the second and third ligatures, and this portion of the trunk is sutured to the liver fascia by means of a vascular needle. Thus, the actual catheterization technique eliminates the necessity of a special ligation of the hepatic duct. The technique is more convenient if the duct is not long.

Technique 2. First the hepatic duct is ligated and separated from the intestinal trunk. Then the latter is cut through between two ligatures but it is separated from the caudal vena cava in a small area. The catheter tip is inserted as described in technique 1, but in this case the passage of the catheter within the lymphatic trunk is discontinued at 1.5–2.0 cm from the corner formed at the site of trunk transition from the pancreas parenchyma to the ventral wall of the caudal vena cava. To stabilize the catheter in the trunk, the trunk is made to lie down in its bed and is fixed by ligature ends, which have not been cut before, behind the lavsan collar of the catheter.

Technique 3. Figure 4 demonstrates the third technique: the trunk is not cut between the ligatures, it is lying in its bed. Four ligatures are made under the trunk without its separation from the vein wall. The first ligature (a) is

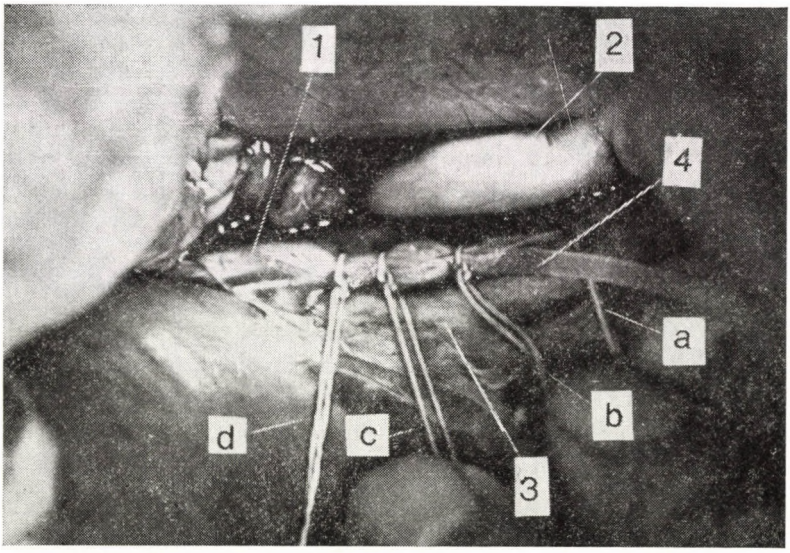

Fig. 4. Catheterization of the intestinal lymphatic trunk. The lymphatic trunk (1), the left renal vein (2), the caudal vena cava (3) and the lymphatic catheter (4) are shown. The first ligature (a) is provided to close the lymph flow and three other ligatures (b, c, d) are placed to stabilize the catheter within the trunk

tightly tied to allow filling of the trunk with lymph and to facilitate the insertion of the catheter (4) by means of a stylet. After the catheter has been positioned, it is fixed by further three ligatures.

Catheterization of the caudal vena cava is performed directly through its own wall or through the wall of the right renal vein. The topography of this area is extremely complex. The anterior edge of the right kidney is strongly pressed into the respective foveolus of the caudate process of the liver. Spreading from the kidney towards the liver, the right suprarenal gland lies on the ventral side of the caudal vena cava, not far from the entry of the caudal vena cava into the liver. In the same area, somewhat medially, the coeliac artery originates from the abdominal aorta; the hepatic lymph duct joins the intestinal lymph trunk running in a caudodorsal direction towards the cisterna chyli. The whole vascular–lymphatic plexus is located at the level of the anterior edge of the right kidney. Only in a small area (10–12 cm in length), between the anterior edge of the right suprarenal gland and the entry of the caudal vena cava into the liver, is the right side of the vein free: vessels do not enter it. The wall of this portion of the vein is fairly elastic and thick. To gain access to this part of the vein wall, the operator should carefully prepare the caudate process of the liver, a part of which, lying on the fold of the abdominal wall, is adjacent to the pancreas.

Direct catheterization of the caudal vena cava. For the catheter insertion the vein wall is partially cut or punctured with a stylet at the implant site. Priority should be given to the second approach. We called this "non-suture technique for the catheterization of magistral veins". A stylet is introduced into the catheter, and a lavsan collar is fixed on the latter. The vein wall is punctured by the stylet point, and the catheter tip is immediately introduced through the hole. Since the catheter is larger in diameter than the hole made by the stylet, the catheter implantation is not accompanied by blood leakage from the vessel; the lavsan collar limits the passage of the catheter within the vessel lumen; besides, it soon becomes a part of the vessel wall, thus stabilizing the catheter.

To insert the catheter into a "magistral" vein, the operator takes the vessel wall with the left hand and then, with the right hand he takes the catheter at a distance of 3 to 4 cm from its tip to be inserted. When manipulating with a stylet, the operator should be careful to avoid any injury of the opposite wall. For stylet insertion, the operator slightly lifts the vessel wall and with the left hand fixes it properly, while with the right hand he punctures the vessel wall with a sharp cranial thrust of the stylet and advances the catheter through the hole in a cardiac direction. The stylet point should be sharp enough to puncture the wall by the first attempt made. The stylet is stabilized in the catheter with the help of a Pean's forceps so that the stylet point is outside the catheter tip for 3 to 4 cm of its length. If repeated

attempts are made with the stylet, the vessel wall can be punctured in several points, and the air can enter the vein. By means of the stylet the catheter is inserted into the caudal vena cava through the right renal vein. The latter is large enough in diameter and is thin-walled. The catheter is quickly advanced through the lumen of the caudal vena cava without causing any change in the blood outflow in the renal vein.

As the vein wall has been punctured, the operator stabilizes the catheter between the thumb and index finger of his left hand, compresses slightly the caudal vena cava by the middle finger of the same hand to slow down the blood flow, while the assistant removes the stylet from the caval catheter, places the forceps on the latter and, immediately connecting the catheter tip with a syringe, draws off a portion of the blood. Then the catheter is flushed with saline, and its free end is closed with a plug. At this very moment, the operator advances the indwelling catheter with the right hand at a distance of 10 to 15 cm from the lavsan collar (Figs 3 and 4), and holding the vein at the implant site, smoothes out the collar. In the course of the whole procedure haemorrhage is not usual. If it occurs at the implant site, the lavsan collar is pressed to the vein by 1 or 2 laparotomy pads, which are removed just before the closure of the operative wound. Finally, crystalline antibiotics (penicillin, bicillin) are applied to the catheterization sites of both the lymphatic and venous catheters.

Formation of the lymph–caval anastomosis. The scheme of the location of catheters in the intestinal lymphatic trunk and the caudal vena cava is shown in Fig. 5. The lymphatic and caval catheters are exteriorized through stab incisions in the abdominal wall, in the caudal upper end of the operative wound, the former being located above the latter (Fig. 6A). Such a location is convenient for the anastomosis formation, although the inverse location of catheters will not affect the whole process of anastomosing. The distance between the catheter locations in the abdominal wall should not be less than 6 to 8 cm, otherwise, instead of a normal connection of catheters, the anastomosis acquires a form of an acute angle, which disturbs lymph flow.

An incatheter is introduced into the caval catheter so that the former should be in the vessel for 3 to 4 cm of its length with its free end outside the catheter. The intestinal lymphatic trunk catheter is joined up to the caval incatheter by means of a rubber coupling or by inserting the catheter tip into the enlarged tip of the caval incatheter. In the latter case the enlargement should serve as an obturator for coupling of the caval catheter on the one hand and as a bridge for the syringe on the other. The caval incatheter should correspond to the lymphatic catheter in diameter.

Postoperative care. On the second day after the operation, the experimental animal is in good condition and resumes eating; on the third day the animal completely consumes the diet. As a result, lymph clotting in-

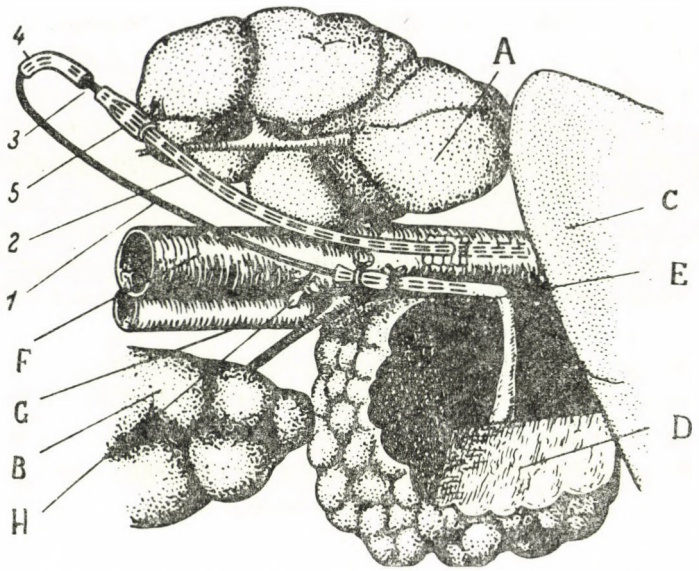

Fig. 5. Scheme of the lymph–caval anastomosis. A, the right kidney; C, the liver; D, the pancreas; E, the intestinal lymphatic trunk; G, the abdominal aorta; H, the distal end of the lymphatic trunk. The parts of the anastomosis are: the lymphatic catheter (1), the caval catheter (2), the incatheter of the caval catheter (3), the rubber tubing (4) and the coupling (5)

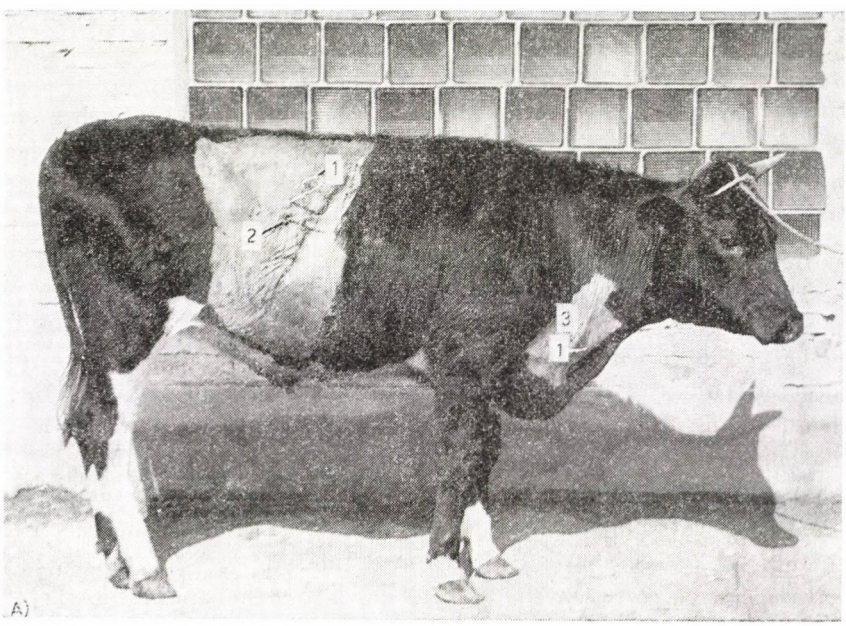

Fig. 6A. A heifer with lymph–caval anastomosis (1), the portal vein catheter (2) and the brachiocephalic arterial trunk catheter (3)

creases. Therefore, the main principle of the postoperative maintenance of the lymph–caval anastomosis is a daily control (3 to 4 times) of its function. For this purpose, the anastomosis is disconnected, the incatheter is flushed with 2.5% sodium citrate solution with penicillin in it, and the anastomosis is restored. Once a day, the indwelling incatheter is removed to be flushed with heparinized saline. The blood is aspirated through the caval catheter, which is then loaded with heparinized saline, and the anastomosis is restored.

It would be possible to catheterize the caudal vena cava and to make the lymph–caval anastomosis without using any incatheter. But in such a case, the lymph would clot within the venous catheter, and the experiment on the animal would have to be discontinued much earlier than in the case when the lymphatic catheter is anastomosed to the caval incatheter.

Lymph collection. The lymph–caval anastomosis is disconnected, the lymphatic catheter end is inserted into a graded flask, the caval incatheter is flushed with heparinized saline and is closed by a plug (Fig. 6B). The rate of lymph flow is measured for 5 to 10 min, and the lymph is collected. Then the anastomosis is restored.

Thus, the above-described techniques allow us to study the intestinal lymph in healthy animals throughout an experimental period of 2 to 3 weeks. However, it should be noted that the study of the intestinal lymph alone is

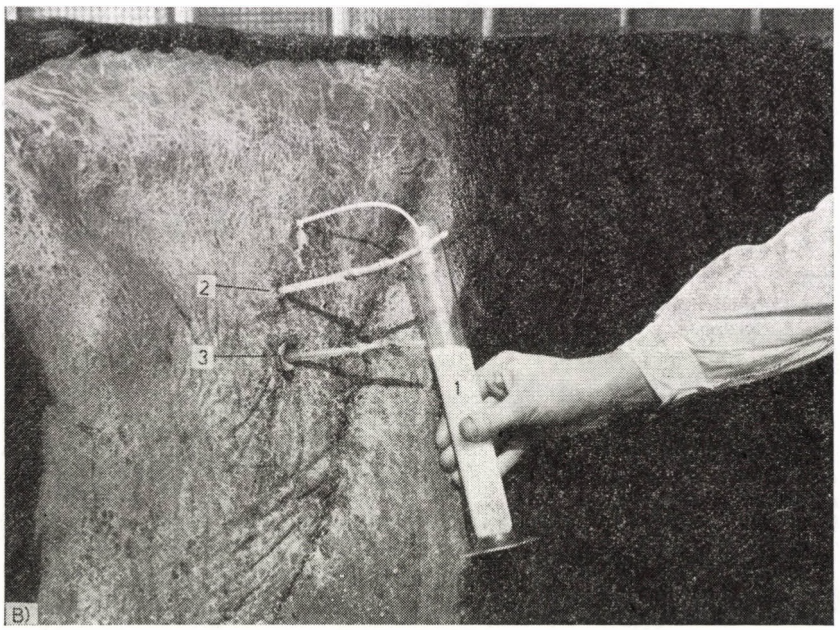

Fig. 6B. Lymph collection (1). The incatheter of the caval catheter (2) and the portal catheter (3) are plugged

not enough for the complete characterization of the metabolism of substances in the digestive tract. As a rule, we use a combination of the lymph–caval anastomosis formation with the catheterization of the brachiocephalic arterial trunk and portal vein (Aliev, 1966).

Discussion

Till the 'sixties researchers virtually failed to carry out experiments like those described in the present paper without facing lymphorrhoea. Experimental surgery of the lymphatic system began to develop progressively with the introduction of polymeric substances. The materials for the catheters, the lymphatic in particular, should be tight, soft, elastic, flexible, and resistant to breakage, in order to ensure a good fixation of the implanted catheter and its long function without any crack or bend. All this is very important, since the intestinal lymph is deep in the body (approximately 30 cm from the body surface), and the catheter manipulated with is to change its direction frequently. The inner surface of the catheter should be perfectly smooth, resistant to moistening and nonthrombogenic. The material should be antiseptic, nonabsorptive and nonelectrostatic. A very important property of a lymphatic catheter is its transparency, allowing visual observation of the lymph flow. Finally, the polymeric materials used for catheter fabrication should be inert and should not be harmful to animal tissues.

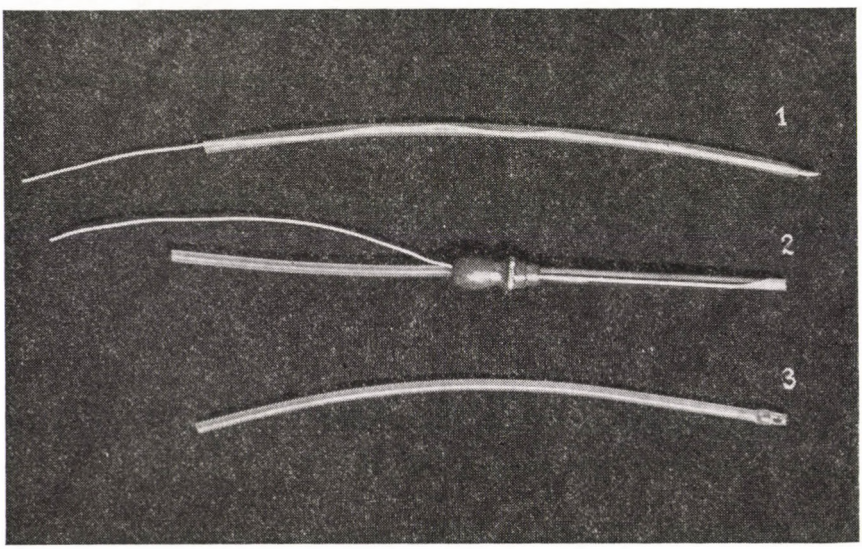

Fig. 7. The stylet (1), the injection needle (2) and the lymphatic catheter (3). The catheter tip has a ball-like inflation and a side hole. T, thread attached to the stylet or the needle for their withdrawal following insertion of the catheter into the lumen of the lymphatic trunk

Substances like polyethylene, polyvinyl, teflon and silicone rubber more or less meet these requirements. In the U.S.S.R., silicone rubber tubings are widely used. We have been utilizing them in experiments for 17 years.

The lymph–caval anastomosis consists of 5 parts: supplying (1) and receiving (re-entrant) (2) catheters and their connections with the lymphatic trunk (3), with venous vessel (4) and between themselves (5). The duration and effectiveness of the anastomosis function depends upon several factors of physiological or mechanical character. The mechanical factors are: inner diameter, smoothness of the inner surface and the length of the catheter, principle of anastomosing or rather creating an external re-entrant bridge; physiological factors: the tension of the lymph in the supplying part of the anastomosis and the sucking activity of the venous vessel to which the tip of the receiving catheter is joined. A long practice has shown that the inner diameter of the lymphatic and blood vessel catheters should not be less than 1.5 to 2 mm. Catheters with an inner diameter more than 3.5 to 5 mm are quickly thrombosed as a result of the low lymph pressure and the slow lymph within the anastomosis. Catheters of 1.5 to 2.0 and 2.5 to 3.0 mm inner diameter are considered to be the most suitable for sheep and cattle, respectively.

The period of the anastomosis function is highly dependent upon the structure of the catheter tip which is to be inserted, and upon manipulations for catheter insertion into the lymphatic trunk. Since it is often impossible to introduce the catheter tip in an "end-to-end" manner, it is to be introduced through the wall of the trunk, i.e. in an "end-to-side" manner. Insertion of the catheter into the lymphatic trunk lumen and its stabilization are the most crucial moments of the operative procedure, requiring much effort and inventiveness of the operator; the quickness and accuracy of the operative manipulations are also of great importance. Ruptures of the lymphatic trunk and surrounding tissues result in an immediate lymph clotting within the anastomosis lumen. To avoid this, a precise knowledge of the topography is needed. Often, the use of a stylet, well-adjusted to the implanted catheter, contributes to the success of operation. Also, an injection needle, with the catheter inserted through it (Fig. 7), is useful. The tip of the needle and stylet should be short enough (not more than 5 cm) to be suitable for the operator to hold and, on the other hand, it should not hinder the access to the lymphatic ducts, especially to those of organs lying in the abdominal cavity. The securing thread helps the assistant to withdraw the above-mentioned devices at the moment when the catheter tip has already been inserted by the operator into the lumen of the intestinal lymphatic trunk. The nozzle of the catheter is of great importance, too. It should be thin-walled enough to provide the desired diameter of its lumen without pressing the vessel wall. Furthermore, it should not be compressed, broken or tightly bound when being ligated. The end of the nozzle should be cut like a tip of a ball-point

pen, and it should be well polished. The bevelled end of the nozzle is not admissible since the thin vessel wall can be easily ruptured; besides, the point of the bevelled end contributes to thrombus formation. The optimal period of functioning of the anastomosis (15 to 18 days) is observed when a special nozzle is used. This has a ball-like inflation, which facilitates catheter stabilization, and a side hole providing better siphonage of the catheter. It is recommended to use a teflon nozzle for a silicon rubber catheter (McGuilliard, 1972). The hardness of teflon ensures the stability of the lumen of the nozzle when the latter is fixed in a lymphatic duct, and the flexibility of the silicone rubber allows catheter exteriorization without any break or compression.

The process of ligature placement and their fixation following the insertion of the catheter into the duct is a further crucial moment of the catheterization technique. The ligature should be placed exactly on the duct. When tying it, the operator should check the thread pressure upon the catheter wall to avoid rupture of the duct wall and a tight binding of the catheter nozzle. When one or two ligatures are made on the duct, with the catheter nozzle being also ligated, the other ligatures (peripheral) can be placed, with the surrounding tissues being sutured. But such an approach should not cause dislodgement or overwinding of the duct. The stabilization of the catheter is accomplished by putting a lavsan collar on it. The collar is soon adhered by fibrin to the duct adventitia and the surrounding tissues. The ligatures virtually lose their stabilizing effect 3 to 4 days after the operation, so the catheter tip is stabilized, owing to proliferation of the surrounding tissues. The lavsan collar contributes to this process, ensuring reliability of the anastomosis work.

The tip of the re-entrant catheter should be inserted into the venous vessel only by means of a stylet. The vessel wall is punctured by the stylet point. Since the diameter of the puncture is less than the external diameter of the catheter tip, blood leakage can be prevented. The latter effect is also provided by the use of the lavsan collar which, being put on the catheter, occludes the vessel lumen on the one hand, and contributes to tissue proliferation on the other.

Now, the connections between the lymphatic catheters are to be discussed. It should be noted that a great number of devices have been specially devised for this purpose, but all these overload the anastomoses and, in some cases, result in dirt accumulations complicating the disconnection and reconnection of the anastomosis. A long practice has proved that the connection of catheters in an "end-to-end" manner by means of a rubber coupling with the consequent fixing ligation is preferable. If the cut ends of catheters are well polished, they adjoin closely, forming a lumen continuity which connects a lymphatic duct with a venous vessel. In this case, a suckling effect of the latter is expressed well enough. Sometimes it is reasonable to make a small

space between the catheter ends to be connected. A frequent palpation in this place assists the fluctuation in the anastomosis system, thus preventing the anastomosis from being blocked and stimulating the lymph flow in the lumen.

With a long experimental use and frequent disconnections and reconnections of the anastomosis, the ends of catheters, especially those of the supplying catheter, will be crushed; sometimes, cracks appear in the catheter wall. To overcome this problem, small rubber collars are put on the catheters to hold them during the manipulations.

The length of catheters should correspond to the location depth of the internal catheter tips within the animal's body. Further, the distance between the skin surface and the contact points of catheters, the so-called height of the anastomosis, should also be taken into consideration. To avoid formation of an acute angle in the anastomosis and to provide a convenient experimental work of the latter, the supplying catheter should be located above the receiving one, and the distance between the sites of their exteriorization should not be less than 6 to 8 cm. The anastomosis acquires a streamlined round form without angles or breaks.

The function of anastomosis should be carefully controlled by palpation. A warm anastomosis means that its permeability is good; a cold anastomosis should be attempted to disconnect immediately. The high rate of the lymph flow at the moment of anastomosis disconnection indicates that a blockage of the receiving catheter has occurred. In that case, the incatheter is withdrawn, flushed with anticoagulant and is replaced into position, or it is simply replaced by a new one. No lymph flow indicates blockage of the whole system or the supplying catheter only. Sometimes it is possible to remove the fibrin column from the supplying catheter to make the lymph move.

The use of anticoagulants for maintenance of the anastomosis function should be limited; otherwise, it distorts the physiological condition of experimental animals. Moreover, since the lymph, the intestinal lymph in particular, has been studied in relation to the lipid metabolism by most researchers, the use of heparin is excluded for known reasons. Heparin should be used in a concentration of 100 to 150 units/ml saline with penicillin (1 million units/100 ml) added to load the catheters of blood vessels and to flush the re-entrant catheter of the lymph–caval anastomosis when, for example, lymph collection is stopped for a given day and is taken up again the next day, or later. During lymph flow measurements the supplying catheter is disconnected and immediately introduced into a supplying flask, while the re-entrant catheter is loaded with 2 to 3 ml of 2.5% sodium citrate solution and closed with a polyethylene plug. After 5 min of lymph collection, the anastomosis is restored. It should be noted that a good physiological condition of surgically prepared animals and an excellent function of the anastomosis are observed when animals are used in the experiment daily.

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BOOK REVIEWS

Hans G. NIEMAND and Peter F. SUTER (eds): *Praktikum der Hundeklinik.* Sixth, fully revised edition. Verlag Paul Parey, Berlin und Hamburg, 1989. 827 pages, 375 illustrations including 49 illustrations in colour, 97 tables. Price: 198.— DM. ISBN 3-489-50816-5.

This book, the first edition of which was published under the editorship of *Hans G. Niemand* in 1961, has run into six editions so far. The rapid succession of editions indicates the keen interest taken in the subject and the success and favourable reception of the work.

The book is the work of an authors' collective. Now for the first time, the sixth, fully revised edition was published under the editorship of Professor *Peter F. Suter* who wrote many chapters of the book.

The arrangement and contents of the book are as follow.

In *the general part*, Chapter 1 entitled "*Practice and clinic*" (H. G. Niemand) deals with the up-to-date equipment and instrumentarium of the small animal clinic and hospital, and with the methods of drug administration.

Chapter 2, "*Nutrient supply*" by *W. Drochner* describes the nutrient requirements of dogs and the dietetic nutrition as well as dietary regimens.

Chapter 3 is entitled "*Clinical examination of dogs*" *(H. G. Niemand).*

Chapter 4, "*Laboratory examinations*" *(R. H. Gwalter)* gives an excellent and sufficiently detailed description of up-to-date laboratory methods which can, and must, be used by a veterinary practitioner aiming at high-standard work. After describing the laboratory equipment and installations necessary for veterinary practitioners, this chapter sums up the important laboratory methods for haematological examinations, urine analysis, faecal examination, and analysis of the liquor and the pathological body fluids.

Chapter 5, entitled "*Veterinary documents, case histories, certificates*" *(P. F. Suter)* gives a description of the written documents associated with veterinary practice.

Chapter 6 is entitled "*Analgesia, sedation, anaesthesia*" *(R. Skarda).*

The *special part* of the book begins with Chapter 7 which discusses "*Hereditary diseases and predisposition to diseases*" *(H. G. Niemand).*

Chapter 8 is an up-to-date and thorough summary of "*Diseases of the immune system and immunopathies*" *(P. F. Suter).*

Chapter 9, "*Dogs injured in accidents*" by *P. F. Suter, J. Arndt, W. D. Prieur, G. Kása* and *F. Kása*, is a very important part embracing a large body of knowledge. It describes the examination of dogs that have suffered an accident, the fundamental elements of giving first aid, then discusses at length the development and therapy of shock. This is followed by the description of injuries of the respiratory organs, abdominal organs, nervous system and organs of locomotion, and possible ways of treating these injuries.

Chapter 10 is entitled "*Infectious diseases*" *(P. F. Suter)*, and it offers a brief description of the most important viral, bacterial, protozoal and fungal infections of dogs.

In the general part of Chapter 11, "*Dermatopathies*" by *B. Bigler*, the methods of examination of the skin and the principles of treatment are described. The special part of that chapter discusses skin disease cases of bacterial, fungal, parasitic, allergic, endocrine and metabolic origin, as well as those resulting from environmental noxae.

Chapter 12 bears the title "*Otopathies*" *(P. Sterchi).*

Chapter 13 deals with "*Ophthalmopathies*" *(I. Walde).*

Chapter 14, "*Respiratory organs*" by *P. F. Suter*, begins with the description of the methods for examination and radiological examination. This is followed by diseases of the nose, nasal sinuses, pharynx, larynx and trachea, and finally by diseases of the deeper respiratory passages, the bronchi, the lungs and the pleura.

Chapter 15 is entitled "*Organs of circulation*" *(U. Karsten* and *P. F. Suter)*. First it deals with cardiological diagnosis and gives a sufficiently detailed description of electrocardiography (ECG). This is followed by diseases of the heart, pericardium, blood and lymphatic vessels.

Chapter 16, "*Anaemia and haemorrhagic diathesis*" *(P. F. Suter)*, first tackles the problems of clinical and laboratory diagnosis as well as pathogenesis. This is followed by questions associated with the therapy of anaemia, with special respect to blood transfusions and how to perform them. This chapter deals with the disorders of haemopoiesis, haemophilia, the disorders of blood clotting, and diseases associated with platelet deficiency.

Chapter 17, entitled "*Qualitative and quantitative changes in leukocyte count and diseases of the leukopoietic system (P. F. Suter and Chr. Saar)*, evaluates pathological changes in white blood cell count. In this chapter are discussed diseases like reticulosis, leukosis and diseases of the spleen.

Chapter 18 is entitled "*Digestive organs*" *(P. F. Suter, H.-O. Schmidtke* and *Ch. Uehlinger)*. Its introduction presents the diagnostic evaluation of anorexia, swallowing difficulties, salivation, vomiting, and diarrhoea. Subsequently the examination and diseases of the oral cavity, teeth, tongue, salivary glands, tonsils, pharynx and oesophagus are discussed, together with diseases of the stomach, the aspects and consequences of ileus and diarrhoea, and enteritis of bacterial, toxic, parasitic and viral origin. Surgical diseases of the intestine, e.g. hernia, are also dealt with. To this chapter belongs malabsorption syndrome, maldigestion, and digestive trouble due to pancreatic insufficiency.

Chapter 19, "*Diseases of the liver and biliary ducts, ascites*" *(P. F. Suter)*, describes the clinical signs of hepatic insufficiency and its diagnosis by laboratory methods. This is followed by principles of the therapy of hepatopathies, questions associated with icterus, and diseases and pathological lesions of the gall-bladder and biliary ducts. Inflammatory and toxic hepatopathies, hepatic cirrhosis, fatty degeneration of the liver and the consequent abdominal dropsy (ascites) are also dealt with in this chapter.

Chapter 20 bears the title "*Peritonitis and pancreatitis*" *(P. F. Suter)*.

Chapter 21, entitled "*Urinary system*" *(P. F. Suter)*, begins with the description of the general symptoms of nephropathies, evaluation of changes in urine volume, urine analysis, kidney function tests, and special methods for diagnosing renal diseases. This is followed by questions of chronic and acute renal insufficiency and the different renal diseases. Among diseases of the lower urinary passages the diagnosis and treatment of urolithiasis are highlighted. This chapter also contains a description of urination disorders.

Chapter 22, "*Male genital organs*" *(P. Sterchi)*, deals with the collection and examination of semen, the disturbances of sexual activity, and the pathological changes of the testicles and accessory sex glands.

Chapter 23, "*Female genital organs*" *(H. Gehring* and *S. Arnold)*, begins with an anatomical and physiological overview and a description of physiological and pathological sexual activity as well as of the special methods of examination. This is followed by the description of artificial insemination, pregnancy diagnosis, and reproductive disorders of bitches. Physiological and complicated whelping and assistance at whelping are also described. This chapter also deals with puerperal disorders, the disorders of sexual activity, the pyometra—endometritis complex, and other diseases of the female genital organs. Finally the diseases of the external genitals and mammae are summed up.

Chapter 24 is entitled "*Endocrinopathies, diseases of the endocrine glands*" *(P. F. Suter)*.

Chapter 25 bears the title "*Organs of locomotion*" *(W. D. Prieur, G. Kása* and *F. Kása)*. It deals with metabolic, inflammatory and hereditary diseases of the skeleton, the joints and the muscles.

Chapter 26 is entitled "*Diseases of the nervous system*" *(J. Arndt)*. It describes the neurological examination, the special methods of examination, the general nervous symptoms, and behaviour disturbances. Subsequently the most important diseases of the cerebrum, epilepsy, syndromes associated with disorientation and ataxia, and the pathological lesions of the brain stem, spinal cord, and peripheral nerves are dealt with.

Chapter 27 bears the title "*Diseases of the vertebrae*" *(P. F. Suter)*.

Chapter 28 is entitled "*Intoxications and poisonings*" *(H. G. Niemand)*.

Chapter 29 is the *Appendix (P. F. Suter)* which contains useful information about units of measurement and medicines as well as their dosage.

The book begins with a *Preface*, an *Author Index* and *Contents* and ends with a detailed *Subject Index*.

The book covers a very wide field of knowledge. Though written by 19 authors, it creates a uniform overall impression and its chapters are not disproportionate. This can be attributed to the purposeful and painstaking work of the editors. The chapters contain all the knowledge

which may be of interest to veterinarians working in canine practice. This body of knowledge is presented in a clear and concise style, with a scientifically well grounded and practical approach.

While maintaining the guiding principle of the earlier editions, that is the effort to meet the requirements of small animal practitioners, in its contents the sixth edition has been noticeably modernized. More space is devoted to the aetiology, and mainly to the pathogenesis, of diseases and the diseases of the different organs are discussed in more detail. The excellent description of up-to-date instrumental and laboratory methods should be pointed out, as well as the proposals for their possible uses in harmony with the practical requirements and possibilities. The reader gets abundant information on the manner of treatment of dogs, the examination of patients, the examination of different organs by special methods, and the interpretation of the clinical signs with respect to differential diagnosis.

In summary, the book gives a very favourable impression. It is a well-edited, high-standard, practicable and interesting contribution to veterinary literature. It contains all the up-to-date knowledge which may be necessary for a general practitioner dealing with canine practice, and is a manual indispensable for the everyday activity of veterinarians working in this field.

The Reader receives a book written in highly readable style, having first-class typography, beautiful get-up and exquisite binding. The book contains a large number of high-quality illustrations. This work, published under the auspices of *Verlag Paul Parey* and representing the high standard usual for works published by that publishing house, will surely be highly successful and raise the reputation of its publisher.

Ferenc KARSAI

Norman F. CHEVILLE: *Introduction to Veterinary Pathology*. Iowa State University Press, Ames, Iowa 50010, U.S.A., 1988. First edition, 548 pages, hardcover, $ 59.95.

Owing to the accelerated exchange of knowledge and the heavy responsibility resting on those dealing with specialist training, a newly published technical book always arouses keen interest. This is especially true for a book tackling such a complex subject as pathology, primarily general pathology, which requires a thorough knowledge of a rather wide range of fields. One of the most difficult (and most beautiful) tasks of university education is to elucidate the complex interaction between the disease-causing agents and the living organism, that is the essence of disease. In the past decade textbooks on general pathology were published, among others, in German and English. These are now followed by this comprehensive work by *N. F. Cheville*, a renowned expert whose book entitled "Cell Pathology" was published in its second edition in 1983.

The author discusses the subject of general pathology in 7 chapters including a total of 30 subchapters.

Chapter 1 (Introduction) discusses the pathogenesis of disease, the response of the organism to the disease-causing agents, and makes clear some fundamental concepts (degeneration and necrosis, circulatory disturbances, inflammation, growth disorders, neoplasm). Among the main factors which can upset the homeostasis, the author discusses in detail the oxygen and water deficiency, the disturbances of inorganic ion and acid-base balance, the end-products of protein metabolism, and the development of uraemia. This chapter is completed by a part in which the main aspects of death, the postmortem changes and postmortem examination are discussed. This introduction offers a brief survey of the whole book and of questions which will be discussed in later chapters more lengthily. E.g. necrosis is mentioned in two subchapters of the Introduction. For a beginner this system of arrangement perhaps makes it a bit difficult to orientate.

Chapter 2 embraces the subject of degeneration, including the general phenomena and cytopathology of cellular degeneration, the role of cell organelles and their changes during pathologic processes, the disturbances of carbohydrate, lipid and calcium metabolism, the endogenous and exogenous pigments, and conditions caused by the precipitation of various crystals (oxalosis, uricosis, etc.). A separate subchapter is devoted to processes which take place in the extracellular space (collagenosis, mucopolysaccharidoses, amyloidosis).

Chapter 3 presents the disturbances of growth through cellular changes (hypertrophy, hyperplasia, metaplasia, atrophy, hypoplasia, aplasia, dysplasia) and through the fundamental conceptions of teratology. Three subchapters are devoted to the subject of neoplasms.

Chapter 4 discusses the fundamental changes of the blood and the vascular system (oedema, shock, infarction, embolism, cardiovascular disorders, haemostasis, thrombosis, anaemia).

Chapter 5 deals with inflammation and healing, *Chapter 6* discusses problems of immunopathology, and *Chapter 7* lists the disease-causing factors (physical, chemical and biological agents, nutritional factors and genetic defects).

The author discusses the problems of general pathology with an up-to-date approach, using the newest achievements of molecular biology and pathophysiology and demonstrating also the ultrastructural changes. The book, which is conscientiously based on a functional pathological approach, will be an invaluable aid to modern veterinary education. Less space is devoted to gross morphological descriptions: this is understandable, as these can be learnt more easily in the framework of practical training. Attention is called to the most important aspects by using subtitles.

The text is accompanied by excellent illustrations including photographs of gross pathological lesions, light and electron micrographs, schematic drawings, and by tables. At the end of each chapter, under the title "Focus", there is a clinico-pathological description of a selected disease belonging to the subject discussed in the given chapter. E.g. chronic hypoxia is illustrated by the example of acropachy (pulmonary hypertrophic osteoarthropathy), granulomas are illustrated by the example of tuberculosis, and septicaemia by that of anthrax, etc. These examples make the book colourful and prepare the readers for using their knowledge of general pathology in the field of special pathology. A reading list is given at the end of the subchapters.

The typographical make-up of the book is excellent. This textbook will not only be an invaluable help to students of veterinary medicine but it will be of use to all those interested in veterinary pathology. Perhaps it would have been helpful to include a list of the abbreviations used in the text.

Andor KARDEVÁN

H. W. FERGUSON: *Systemic Pathology of Fish.* Iowa State University Press, Ames, Iowa 50010, U. S. A., 1989. 276 pages, more than 350 illustrations, hardcover/jacket $ 54.95.

Numerous manuals and textbooks have been published on fish diseases all over the world, and many of them bear the title "fish pathology". These books mostly combine the disciplines of fish culture, epizootiology, parasitology and toxicology. Dr. Hugh W. Ferguson's work can be considered the first book which actually tackles the subject of fish diseases in an approach of classical pathology. As the subtitle implies, this text and atlas presents the responses of teleost tissues, giving a professional pathological description and high-quality illustrations.

The text is divided into 13 chapters. With the exception of the first and the last chapter, the author discusses the pathophysiological and gross pathological lesions by organs and organ systems. Although it is not stated definitely, most disease cases presented are from salmonids, first of all from trouts. As the author mentions it in his preface to the book, most examples are drawn from diagnostic or experimental material processed by the Fish Pathology Laboratory, Ontario Veterinary College.

The second part of Chapter 1, discussing the pathophysiological processes and the host response, can be considered especially interesting and new. The reading list given at the end of this chapter contains only two references, indicating that, apart from the author's own results, the special literature of that subject is very scanty, and that much remains to be done in the field of fish pathology. The other chapters are followed by ample reading lists containing papers not only on trouts and American fish species examined by the Fish Pathology Laboratory, Ontario Veterinary College, but also on disease-causing agents and pathological lesions caused by them in the organs of cyprinids and marine fishes. It is somewhat unusual, though useful to the Reader, that the reading lists contain numerous papers which are not cited in the text.

All chapters contain a large number of illustrations which are in good harmony with the text. In contrast to the majority of other manuals, the illustrations of Hugh Ferguson's work highlight the host response, and within it the tissue and cellular responses, rather than the aetiological agent or type of lesion.

Of the shortcomings, the lack of uniformity can be mentioned. As the book does not present a single definite fish species which could serve as a basis for demonstrating its diseases in a complex manner, less thoroughly trained specialists may find it rather difficult to understand. However, it provides very useful information for those experts who have a fair knowledge of pathology and pathogenic agents of trouts and cyprinids, and wish to go into the subject even more deeply. This book is therefore very much recommended for such experts, primarily for veterinarians but also for other pathologists and for students.

Kálmán MOLNÁR

COMING EVENT

The International Society for Animal Hygiene invites to the VIIth International Congress for Animal Hygiene being held in Leipzig, German Democratic Republic from August 20th to August 24th 1991.

The main theme is "Unity of Animal Hygiene and Livestock Production".

Sub-topics are:

- Ecological fundamentals of modern animal hygiene
- Animal hygiene programmes for prevention and control of epizootic diseases, multifactorial diseases and technopathies
- Disinfection, insect and rodent control in animal houses
- Animal hygiene–environmental protection and utilization of agricultural waste products
- Food hygiene
- Noxious substances in animal houses
- Hygiene of the food chain: soil–plant–animal–man
- Animal welfare–animal health and performance
- Microflora in animal houses and infection pressure
- Evaluation of environmental stability and husbandry systems by means of hygienic and ethological methods.

During the VIIth International Congress two satellite symposiums will be held with the topics

- Animal behaviour as a criterion for animal health
- Academic training and postgradual study of agricultural specialists in the fields of ecology and animal hygiene.

Authors wishing to deliver a paper should send the abstract (max. one typewritten page, English or German) to the Organizing Office of the VIIth International Congress for Animal Hygiene, Semmelweisstr. 4, DDR–7L10 Leipzig, German Democratic Republic by October 15th 1990, at the latest. Questions relating to the congress should be addressed to the Organizing Office.

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Acknowledgement of grants and technical help.

References. Cite only essential references. They should be arranged in alphabetical order of the authors' surnames, without serial numbers. The reference list at the end of the paper should contain

- for journals: names and initials of all authors, year of publication (in parentheses), colon, English title of the paper (if the original title is not English, indicate in parentheses, e.g. [in French]), journal title abbreviated according to the style used in Index Veterinarius, volume number, issue number in parentheses, first and last pages;
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Tables. They should be typed on separate sheets and have a concise heading each. Tables are to be numbered sequentially by Roman numbers.

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DAMAGES TO FEED PROTEINS AND THEIR NUTRITIONAL CONSEQUENCES: A REVIEW

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(Received September 7, 1989)

The consequences of heat processing of feeds for sterilization and to destroy antinutritive substances can be beneficial as well as detrimental. Damages to feed proteins may be primarily explained by complex reactions between proteins and carbohydrates, by interactions between protein molecules and by oxidation. The chemical mechanisms and nutritional consequences of these reactions are reviewed.

Keywords: Protein quality, heat damage, Maillard reaction, oxidative damages.

Animal feeds may undergo many processes including drying, heating, freezing, milling, pelleting, microbial fermentation, extruding, chemical treatments, etc. The primary reasons for processing feeds are preservation, sterilization and to improve their nutritive value, to increase digestibility and to destroy antinutritive substances. However, the consequences of processing can be both beneficial and detrimental. The concomitant effects of processing (primarily those of excessive heat treatment) may include losses of nutrients, decomposition of biologically active molecules, and impairment of the organoleptic characteristics of the feed.

Damage means an impairment of the nutritive value of the feed. As protein feeds are mixtures of proteins and other substances, the damage to all of the nutrients must be taken into account.

Damage may be a consequence of the combinations of various physical, chemical and microbiological processes. However, with a few exceptions, the damage is primarily attributable to heat and oxidation.

The effects of specific processes (heat, freezing, dehydration, ensiling, chemical preservation, alkali treatment, acid treatment, water treatment, enzyme treatment and irradiation) on the overall nutritive value of feeds have been comprehensively reviewed recently by several experts (Rechcigl, 1982, etc.) The present review is focused primarily on possible impairments in protein quality.

1. Effects of heat processing on protein feeds

Heat processing of feed ingredients is commonly used to inactivate heat-labile antinutritive agents (e.g. trypsin inhibitors in legumes), to destroy undesirable microorganisms (e.g. sterilization of animal slaughter by-products) and enzymes (e.g. thiaminase, peroxidase), to reduce moisture content for preservation and storage, to improve palatability and to increase digestibility (e.g. gelatinization of starches, heat processing of keratins).

Heat is applied for removing solvents (e.g. after fat extraction) or to precipitate proteins (e.g. production of isolates). Heat is also concominant during milling of grains and pelleting of meals.

The effect of heat processing can be *beneficial* as well as *detrimental.* The undesirable effects of excessive thermal processing on nutritive value are, for instance, a loss of heat-labile nutrients, impairment of digestibility and availability of proteins, and loss of palatability.

Adverse effects on carbohydrates and fats need extreme heat processing, whereas proteins have proved to be more susceptible to thermal damage.

Heating proteins generally enhances their digestibility due to denaturation; however, the availability of certain amino acids (primarily that of basic amino acids) may be adversely affected in the presence of reducing sugars as a consequence of the Maillard reaction. Heat damage to proteins can also result from destruction of certain amino acids (methionine, cystine, tyrosine) by oxidation and from modification or formation of intramolecular linkages between peptide chains.

The extent of protein damage may be largely different, depending on the moisture content of the feed (wet or dry heat treatment). The diminutive effect of water or saturated steam may be explained not only by media low in oxygen, but also by differences in the spatial behaviour of the protein molecules in the presence or absence of water.

As a consequence of heating, chemical reactions are accelerated, whereas enzymatic reactions will stop because of protein denaturation.

1.1. Mechanisms of heat damage in proteins

The first consequence of heat treatment of a protein is cleavage of secondary chemical bonds because of an increased heat-oscillation of the molecules and atoms. Besides, disulphide bridges may be destroyed in particular in oxidative or reductive media.

The result of these changes is called *denaturation*, during which the tertiary and quaternary structure of the protein molecule is modified; the primary structure (i.e. the covalent bonds of the protein) remains basically unchanged. Denaturation is regularly an irreversible process, which results

in disappearence of any biological activity (e.g. enzyme activity) connected with the native conformation of the protein.

Denaturation itself may be considered to be *beneficial* from the nutritional point of view, because it makes the protein molecule more susceptible to the attack of digestive enzymes. However, the functional properties of dietary proteins are damaged (e.g. immunoglobulins in milk).

Denaturation was shown to be beneficial for the elimination of the effects of protein-like antinutritive agents (e.g. trypsin inhibitors in soybean). Thus, the nutritive value of legume seeds may be improved by heat treatment, due to destruction of heat-labile toxic substances, but improved digestibility also plays a role.

During excessive heat treatment the primary structure of the proteins may also be damaged by cleavage of peptide bonds and chemical destruction of amino acids. This chemical damage of proteins is named "*deterioration*".

The speeded-up reaction of free amino groups of a protein (most of the free amino groups originate from the epsilon-amino group of lysine residues) with the oxo groups of aldehydes (e.g. reducing sugars) during heat processing is considered to be disadvantageous because of the concomitant loss of nutritive value.

The availability for utilization of epsilon-amino bound lysine will be reduced; however, the total lysine content determined by ion-exchange chromatography after acid hydrolysis of the protein shows negligible change. Thus, determination of total lysine content is an insensitive means of monitoring nutritional damages.

Destruction of amino acids from the chemical point of view may be detected only after extreme heat treatment of proteins. Several types of nutritional damage of proteins have been elucidated: (*i*) reaction between the amino groups of amino acids and a reducing substance (e.g. reducing sugars); (*ii*) reaction between the amino groups of amino acids and a carbonyl group of autoxidized (rancid) fats; (*iii*) protein–protein interaction (formation of inter- and intramolecular bond) independent of the presence of reducing substances; (*iv*) damage to sulphur amino acids by oxidation or desulphydration.

1.2. Reactions between proteins and carbohydrates

Maillard reaction

The fall in nutritive value of proteins during heat treatment in the presence of reducing carbohydrates can be well explained by the Maillard, i.e. nonenzymatic browning, reaction between aldehyde (oxo) groups of carbohydrates and free amino groups of proteins.

The chemical mechanism, biochemistry and nutritional aspects of the Maillard reaction have been extensively studied and reviewed by many authors (e.g. Lea and Hannan, 1949; Mohammad et al., 1949; Donoso et al., 1962; Finot and Mauron, 1972; Freimuth, 1973; Adrian, 1974; Dworschak and Hegedűs, 1974; Dworschak, 1980; Dworschak and Örsi, 1981; Mauron, 1981; Finot et al., 1981; Feather, 1981; Gumbmann et al., 1983; Öste and Sjödin, 1984).

Mechanism of the Maillard reaction

The Maillard reaction comprises the very complex reactions of oxo groups (aldehydes, ketones, reducing sugars, oxidized lipids) with primary amino groups (amines, amino acids, peptides, proteins). In feed proteins most of the free primary amino groups are presented by the epsilon-amino group of lysine residues. Alpha-amino groups of N-terminal amino acids or free amino acids play an almost negligible role.

The Maillard reaction may be divided into three stages: early, advanced and final Maillard reactions. The first step is a chemically well-defined carbonyl–amino condensation reaction withou tbrowning. The second stage refers to the very complex reactions among various derivatives being formed, leading to volatile or soluble substances. At the final stage insoluble brown polymers are developed. Suggested pathways of the Maillard reaction are illustrated in Fig. 1.

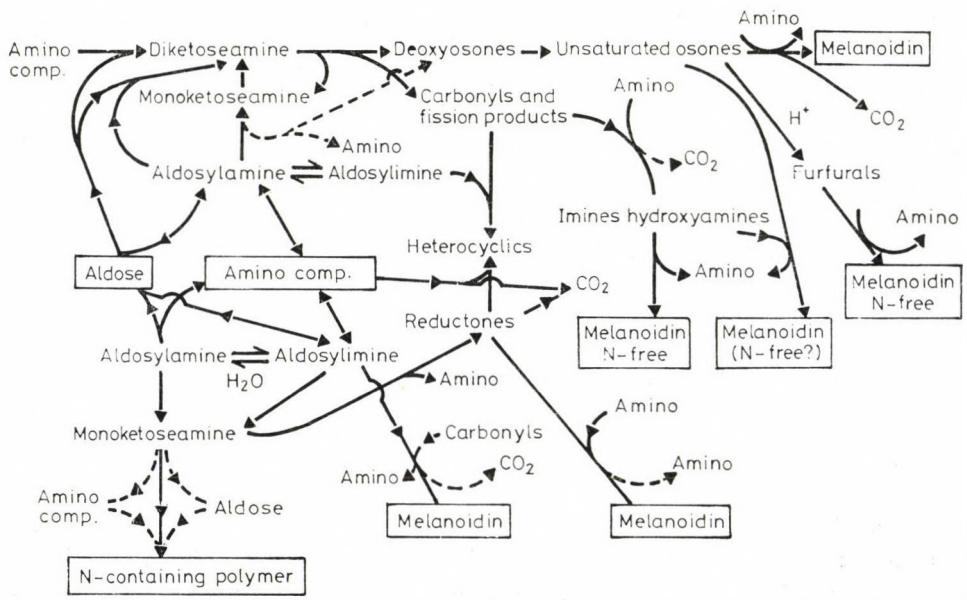

Fig. 1. Pathways of the Maillard reaction (Dworschak, 1980)

Factors influencing the rate of the Maillard reaction

Temperature, duration of heating, water content and pH were all shown to affect the rate of the Maillard reaction in model systems. The effect of temperature on the rate of reaction in casein–glucose mixtures is very pronounced above 80 °C; however, the velocity of the loss of amino nitrogen considerably increases also at lower temperatures during long storage. The formation of brown polymers was shown to augment with the square of the reaction time.

Water is essential for the initial steps; however, it may inhibit dehydration reactions during the advanced stage of the Maillard reaction. Maximum browning appears to occur at about 30% moisture content; however, maximum losses of free epsilon-amino group of lysine residues were obtained at a lower moisture level.

Alkaline pH values accelerate the Maillard reaction by making the basic amino groups more susceptible to the attack of oxo groups having a nucleophilic character.

Nutritional consequences of the Maillard reaction

The nutritional consequences of the Maillard reaction may be beneficial (production of flavours and aroma) as well as detrimental (loss of amino acids, reduction in the availability of amino acids and in digestibility, as well as formation of toxic and mutagenic compounds).

Early studies were concentrated on the fall in the growth-promoting ability of milk powders as a consequence of drying. Milk was shown to be especially susceptible to Maillard reactions. During conventional drying (roller drying) of milk and subsequent storage of milk powder, the free epsilon-amino groups of lysine residues may interact with lactose. The resulting lactosyl-lysine is released on enzymatic digestion, however, it is biologically unavailable to the animal and is excreted in the urine.

The main negative consequence of the Maillard reaction is the fall in available or "reactive" lysine content of the protein. Destruction of other essential amino acids, a reduction of digestibility and biological value (BV) as well as production of toxic substances or metabolic inhibitors may also be observed.

On the other hand, heat treatment (cooking, roasting) of foods or feeds increases their enjoyment and palatability by formation of flavours and aroma due to non-enzymatic browing reactions.

Choice of an assay for monitoring nutritional damage caused by the Maillard reaction

Lysine residues having free epsilon-amino group that can react with fluorodinitrobenzene (FDNB) or other appropriate chemical reagents have been termed "available" lysine. However, this value is not always equivalent to the level of biologically available lysine; therefore, lysine content measured by chemical reagents was proposed to be termed "reactive" (e.g. FDNB-reactive) lysine.

To monitor nutritional damage caused by the Maillard reaction, a proper selection of assays is necessary. The lysine-sugar compounds are split by acid hydrolysis, so that conventional amino acid analysis may reveal only a small loss in the total lysine content.

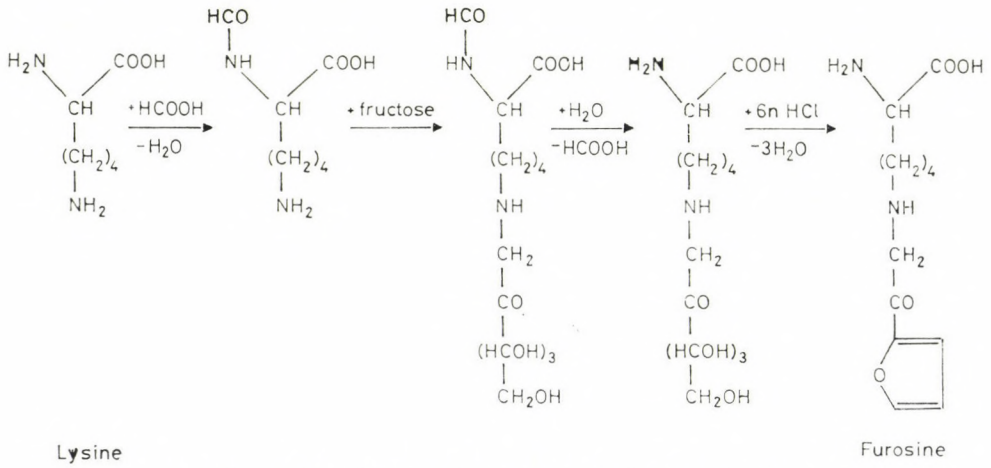

Fig. 2. Formation of furosine from lysine and fructose (Mauron, 1970)

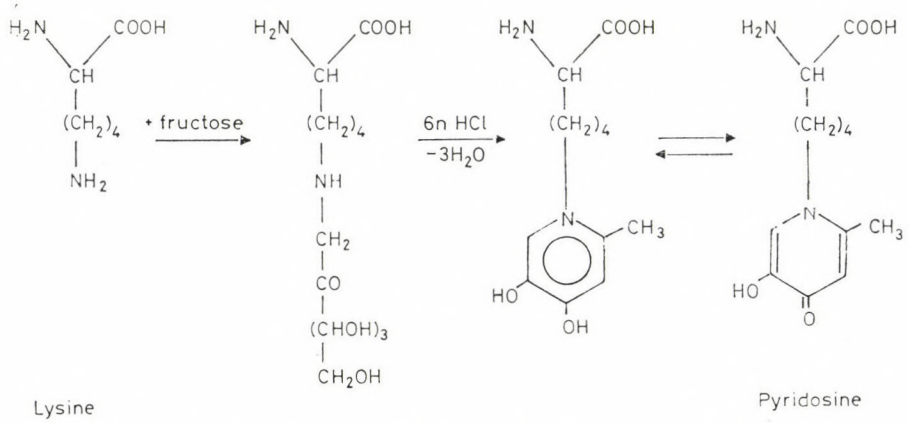

Fig. 3. Formation of pyridosine from lysine and fructose (Mauron, 1970)

In milk powders the losses in the limiting methionine may be of greater concern than the loss of lysine, for milk has a surplus of lysine. Thus, a considerable proportion of lysine may be inactivated without a corresponding reduction in BV or NPU value. The same is true for meat-and-bone meals. However, if heat-damaged milk powders or meat-and-bone meals are evaluated in mixtures with gluten, where lysine is limiting, even a small impairment in the availability of lysine can be easily monitored by BV, NPU, PER or NPR assays, as these biological measurements of protein quality are considered to reveal changes in nutritive value only when these changes affect the limiting amino acid (Hegedűs et al., 1981).

The acid hydrolysis of epsilon-deoxy-lactulosyl-lysine (which is formed by the reaction of lactose with lysine) yields lysine, furosine and pyridosine. Chromatographic separation of furosine and pyridosine may be used to monitor the extent of the Maillard reaction in milk. Furosine and pyridosine may be synthesized from lysine and fructose (Figs 2 and 3).

Reactions between proteins and non-reducing sugars

Sucrose as a non-reducing disaccharide may participate in the Maillard reaction only after inversion, i.e. after hydrolysis of the glycosidic bond releasing glucose and fructose. Direct split of the glycosidic linkage of sucrose by aminolysis with the epsilon-amino group of lysine has been proposed but not confirmed.

1.3. Protein–protein interactions

The nutritional damage in heated proteins may also occur if reducing carbohydrates or other substances having reactive oxo groups are not present. Formation of intramolecular cross-linkages, oxidation of sulphur amino acids, desulphuration and deamination are responsible for this; however, the nutritional damage is less than that in the presence of reducing sugars.

Mechanism of cross-link formations

Several cross-link-bound formations between protein or peptide chains have been suggested by Mauron (1970) (Figs 4 and 5). Glutamic and aspartic acids may theoretically form *imide* linkages with asparagine and glutamine, *thioester* linkages with the thiol group of cysteine, or *ester* linkages with the hydroxyl groups of threonine and serine. Lysinoalanine formation can be expected only at alkaline pH (for instance, in alkali-treated keratins).

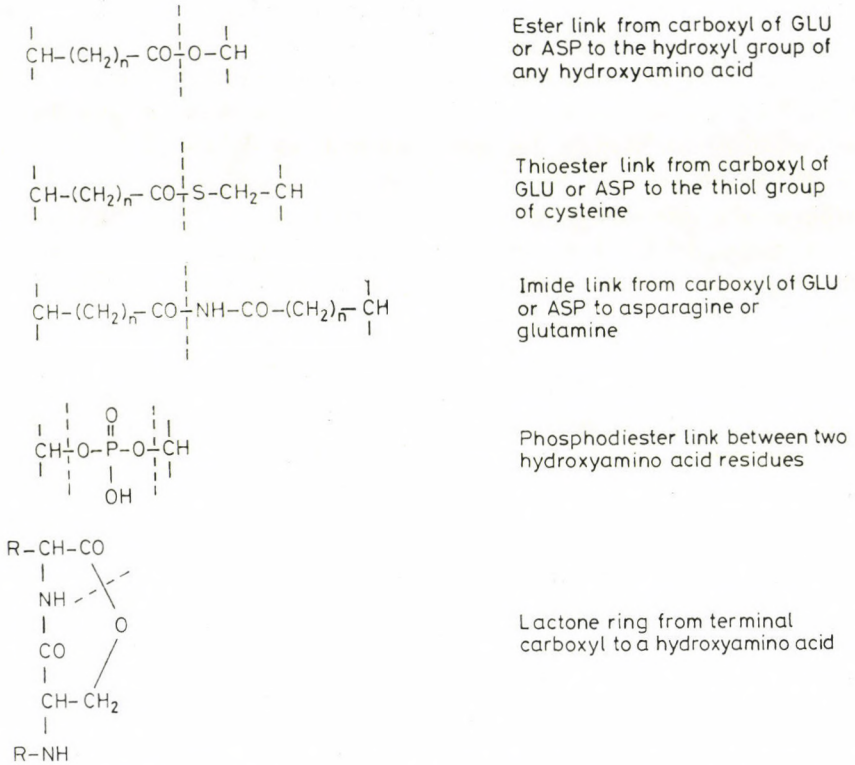

Fig. 4. **Possible cross-link formations between polypeptide chains (Mauron, 1970)**

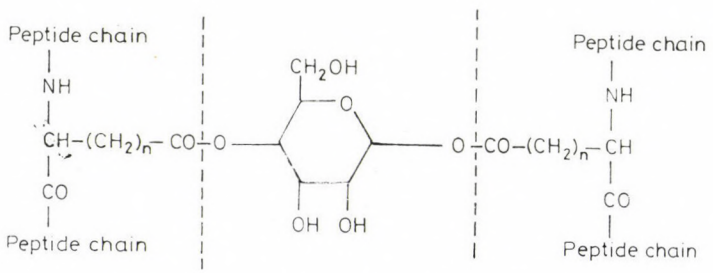

Fig. 5. **Ester cross-link via hexose molecule (Mauron, 1970)**

The formation of unnatural amide linkages between or within protein chains was shown to occur between the free epsilon-amino group of lysine residues and the free carboxyl groups of aspartic or glutamic acids (Fig. 6). However, evidences of liberation of NH_3 have suggested that the amide groups of asparagine and glutamine are more important from this respect (Bjarnason and Carpenter, 1970).

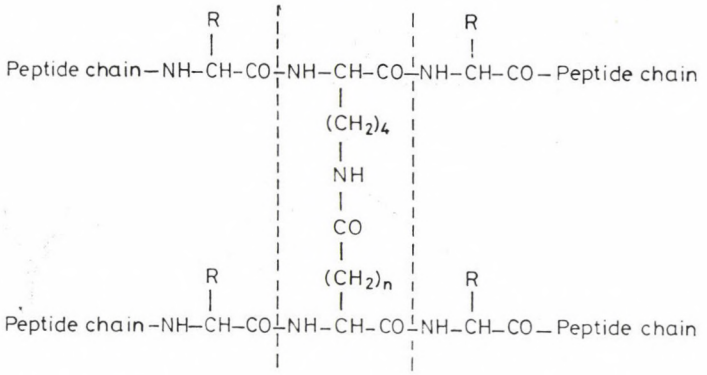

Fig. 6. Cross-link formation between the epsilon-amino group of lysine and asparagine or glutamine (Bjarnason and Carpenter, 1970)

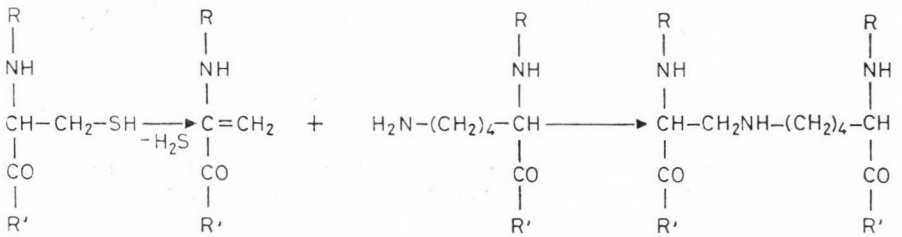

Fig. 7. Cross-link formation between cysteine and lysine with liberation of H_2S (Hegedűs et al., 1981)

The reaction of the free epsilon-amino group of lysine with destruction products of cystine may also be responsible for the loss of availability of lysine and formation of cross-linkages (Figs 7 and 8). The fission of the disulphide bond yields dehydroalanine which may condense with cystine to form lysinoalanine.

Cross-links may also be formed in feed proteins through products derived from protein–carbohydrate reactions.

Nutritional consequences of cross-link formation

Cross-linkages between protein chains may act as physical barriers preventing the penetration of enzymes or altering the sites of enzyme attack.

The nutritional effects of cross-link formation were extensively studied by Carpenter and his coworkers (Bjarnason and Carpenter, 1969 and 1970; Waibel and Carpenter, 1972; Hurrel and Carpenter, 1974, 1976 and 1977; Varnish and Carpenter, 1975*a* and 1975*b*).

The general fall in nutritive value in severely heated animal proteins in the absence of sugars can be explained by reduction of the overall digestibility

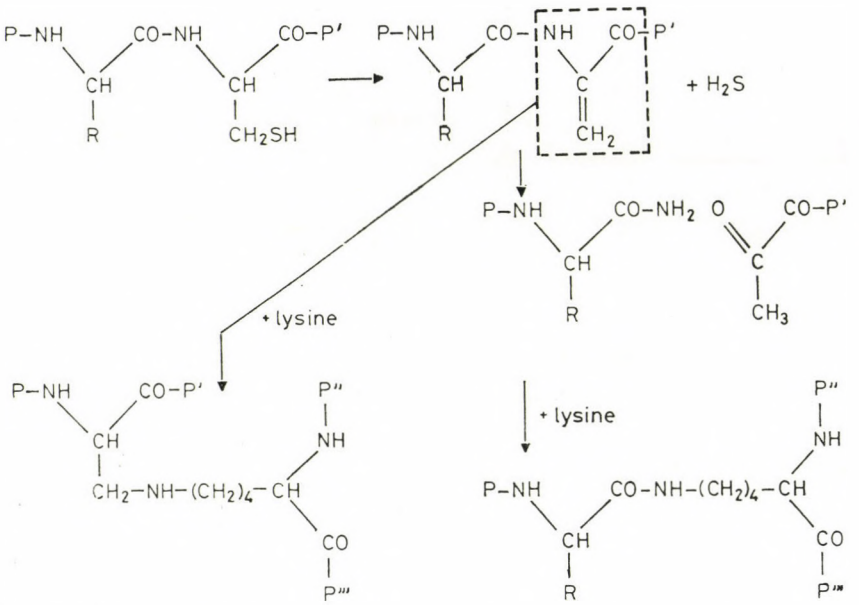

Fig. 8. Possible reactions between the epsilon-amino group of lysine and decomposition products of cystine (Hegedűs et al., 1981)

of the protein, by formation of cross-linkages, though oxidative damage and desulphuration of sulphur amino acids may also occur.

Nutritional, biological, toxicological and medical consequences of cross-link formation, and effects of natural and artificial cross-links on protein structure, reactivity and digestibility were reported recently by Friedman (1977).

Nutritional consequences of epsilon-amino-bound lysine

The alpha-peptide linkages of lysine are hydrolysed in the gut even if its epsilon-amino group is blocked. However, trypsin was shown not to hydrolyse peptides at the carboxyl group of lysine if its epsilon-amino group was blocked. Proteins having lysine residues acetylated or propionylated on their epsilon-amino groups were shown to be digested and absorbed, though considerable quantities of bound lysine were excreted in the urine unutilized (Bjarnason and Carpenter, 1969).

Propionylation of proteins reduced the amount of lysine available for chick growth by about 50 per cent, while the availabilities of methionine and tryptophan were unchanged (Varnish and Carpenter, 1975*a*). Propionylated lysine was shown completely inactive for rat growth, indicating species differences in this respect.

Protein damage by desulphuration and deamination

During excessive heat treatment cystine is decomposed by breaking its disulphide bridge and by liberation of volatile sulphur compounds (H_2S, etc.) by *desulphuration* (Fig. 9).

The liberation of NH_3 during heat treatment of animal proteins results from *deamination* processes (Fig. 10). Formation of intramolecular cross-linkages may be promoted also by ammonia; thus, its liberation reduces the nutritive value of the protein.

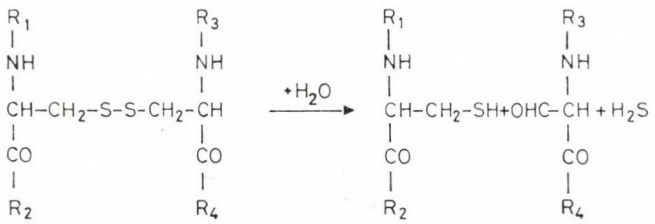

Fig. 9. Decomposition of cystine under wet heat treatment (Bjarnason and Carpenter, 1970)

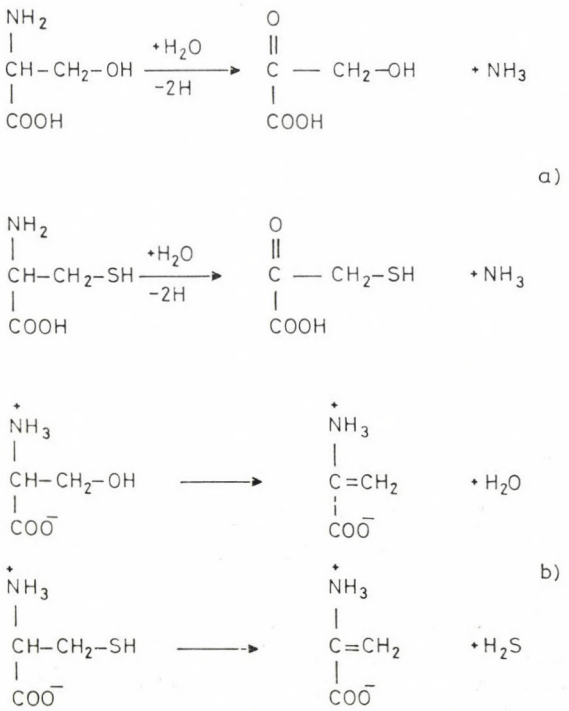

Fig. 10. Decomposition of serine and cysteine via oxidative desamination (a) and beta-elimination (b) (Feeney, 1977)

2. Effects of oxidation on the nutritive value of protein feeds

Oxidative changes in feeds may be developed due to the oxygen content of the air if the specific surface area of the feed particles is extensive. Specific surface is determined by the degree of disintegration of the feedstuff, and it increases quadratically by the diameter of the particles.

Under normal conditions of feed storage oxidative deterioration *per se* plays a minor role. However, at elevated temperature in excess of oxygen, sensitive nutrients (some vitamins, lipids, etc.) may be damaged. These processes may be catalysed by certain heavy metals, light and moisture.

2.1. Oxidation of sulphur amino acids

Damage in protein quality due to oxidation was originally detected in fish meals containing highly unsaturated oils. Model studies with rats on the nutritional value of hydrogen peroxide treated *fish* protein showed a decrease in BV and PER of the protein; however, digestibility was not affected (Sjöberg and Boström, 1977). These changes may be explained by the oxidation of sulphur amino acids.

Methionine may be oxidized to the *sulphoxide* stage during conventional feed processing (heat treatment, drying, storage, etc.), however the extent of it is difficult to determine because methionine sulphoxide is partly reduced to methionine during acid hydrolysis prior to ion exchange column chromatographic separation.

Methionine sulphoxide was shown nearly as available as methionine for rat growth, if sufficient cystine was present (Gjøen and Njaa, 1977). *Methionine sulphone*, on the other hand, was proved to be unavailable for rat growth. However, the oxidation of methionine sulphoxide to *methionine sulphone* requires a more drastic processing, which seldom occurs under the conditions of normal storage of fish meals containing oils rich in oxidized unsaturated fatty acids.

A study on bioavailability of some oxidized derivatives of L-cystine for chicks has shown that *cystine monoxide* and *symmetrical cystine disulphoxide* are nearly as available as L-cystine. However, *asymmetrical cystine dioxide* was found to be only about 50% as available as L-cystine. *L-cysteine-sulphonate* (cysteic acid) was completely unavailable for chick growth (Crawford et al., 1984).

Comparing the efficiency of various sulphur compounds to replace methionine added to a soybean meal diet (10% protein level), *DL-methionine*, *S-methyl-methionine-sulphonium-chloride* and *methionine hydroxy analogue* were shown to be as effective as methionine to improve NPU of the protein (Hegedűs et al., 1988). However, *methionine sulphone* and *methionine sulphoximine*

reduced the NPU value. Inorganic sulphur compounds were shown to be unavailable for replacement of methionine for protein synthesis in the rat (Miller and Samuel, 1970).

2.2. Nutritional consequences of lipid oxidation

Production of slaughter by-product meals by a batch dry rendering process using high pressure and temperature may cause degradative changes in fats or oils. Three types of changes may be differentiated: *autoxidation* at temperatures up to 100 °C; *thermal polymerization* taking place between 200 and 300 °C in the absence of oxygen, and *thermal oxidation* at about 200 °C in the presence of air (Perkins, 1960).

Autoxidation occurs when unsaturated fatty acids of the fat molecule oxidize in the presence of oxygen. This process can be divided into three phases: initiation, propagation and termination. In the first phase initiators (light, heat, traces of heavy metals, peroxides) attack the substrate in the vicinity of a double bond producing highly reactive free radicals. Then free radicals react with oxygen to produce peroxides and hydroperoxides. Peroxides are decomposed to radicals again, which at last form aldehydes, ketones and alcohols. The volatile products are responsible for the rancid taste and odour. At last, stable deterioration products are formed.

Autoxidation is a process which is irreversible but can be retarded. The products of lipid oxidation may interact with many essential nutrients being susceptible to oxidation or to secondary products.

The oxidized fat might be harmful in several ways: the peroxide itself might be toxic, the palatability of feed might be impaired, and certain essential nutrients may be damaged. However, lipids extracted from oxidized herring meal with high peroxide value (142 μmoles/g) caused no growth depression in chicks fed a diet containing over 6% herring oil. A more oxidized fat (peroxide value, 600 μmoles/g) applied at a level of 7% in the diet, however, depressed weight gain to about four-fifths of the normal (Carpenter et al., 1963).

The results of Carpenter et al. (1963) provided no evidence for the toxicity of oxidized fish lipids. Their results have suggested the measures of peroxide value and free fatty acids not to be a useful indicator of the suitability of herring meals for feeding.

A more obvious consequence of lipid oxidation is the concomitant damage of certain essential nutrients.

Essential fatty acids, carotene, vitamins A, E, K and D, pantothenic acid, pyridoxine, biotin, certain amino acids, etc. were all shown to be sensitive to destruction due to peroxides originating from oxidized lipids.

Reactions of proteins with oxidized lipids

Several interactions may occur between oxidized fat and proteins in stored feedstuffs containing highly unsaturated fatty acids (e.g. herring meal). Unsaturated fatty acids may *autoxidize* via free radical reactions into hydroperoxides, peroxides and epoxides, which can degrade into various saturated or unsaturated aldehydes, ketones, hydrocarbons, etc., giving concomitant off-flavours to the rancid product.

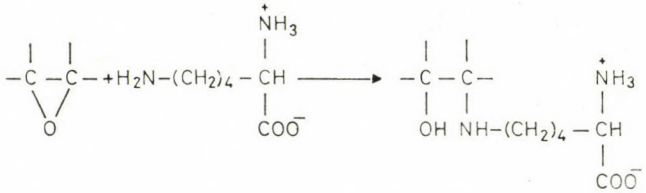

Fig. 11. Reaction between the epsilon-amino group of lysine and the oxirane group of oxidized lipids (Mauron, 1970)

Lipid oxidation products may form *complexes* with proteins during storage, complexes which are partly easy, partly hard to decompose (Pokorny et al., 1968). However, complex formation is not expected to impair the nutritional value of the feed.

Protein damage due to lipid oxidation in autoxidizing lipid–protein mixtures can be explained by *chemical reactions* between lipid oxidation products and side chains of protein-bound amino acids. The most sensitive amino acids have been reported to be methionine, cystine, tryptophan, lysine (Fig. 11), histidine and tyrosine (Nielsen et al., 1985*a*).

Methionine, cystine and tryptophan can be oxidized by peroxides and hydroperoxides of rancid fats, while lysine may react with secondary products of lipid autoxidation (aldehydes). Amino acids with purely aliphatic side chains are assumed not to react.

The extent of reactions is greater at high moisture content, at high temperature, and in the presence of excess oxygen.

Nutritional consequences of reactions of proteins with oxidized lipids

The secondary products of fat oxidation may render the feed *organoleptically* unacceptable, causing a reduction in feed intake and a concomitant reduction in weight gain of the animal.

The oxidation of methionine, due to oxidizing lipids, to methionine sulphoxide seems to be of little nutritional importance, as methionine is regularly highly available to rats. Only a little further oxidation of methionine

sulphoxide was shown to occur to methionine sulphone, which is nutritionally completely unavailable (Nielsen et al., 1985*b*).

Losses in lysine and tryptophan and a general reduction of N digestibility were also reported to explain the slight growth depression of rats in model systems. However, under practical conditions the reactions of proteins with oxidizing lipids are of minor nutritional importance (Carpenter and L'Estrange, 1966; Greenberg and Fraser, 1953; Carpenter et al., 1963; Opstvedt, 1975).

Measurement of the extent of lipid oxidation

The extent of lipid oxidation can be measured by the *peroxide value*, which starts to increase rapidly at the end of the initation phase, then decreases as the formation of stable decomposition products proceeds. The peroxide value measures milli-equivalents of peroxides per kilogram of lipid. Several other tests are based on the measurement of certain compounds produced in the course of the oxidation (e.g. thiobarbituric acid test measures the formation of malondialdehyde). Volatile compounds may be detected by gas chromatography. Free fatty acids are measured by titration with alkali.

The process of autoxidation of fats and lipids in feedstuffs and premixes may be effectively retarded by the use of *antioxidants*, which can act as interceptors of oxygen and free radicals.

2.3. Effect of oxygen on the formation of mycotoxins and polyquinones

Oxidative media were shown to promote mould propagation and mycotoxin formation (Palyusik and Mátrai, 1977). However, development of mycotoxins was inhibited in the presence of excess CO_2.

The oxidation of polyphenolic compounds in plant cells leads to formation of brown colour and polyquinones. These reactions are catalysed by polyphenol-oxidase enzymes that are activated during mechanical crushing of the native plant cells. Polyquinones may be irreversibly bound to the reactive sites of protein, rendering it partly unavailable for digestive enzymes. In reductive, aqueous media (e.g. SO_3 ions) this oxidation may effectively be inhibited.

Conclusions

Overheating of protein feeds during manufacturing impairs the nutritional quality of animal by-product meals, soybean meal, milk powder, etc. The chemical mechanism of the heat damage may be explained by complex reactions between proteins and carbohydrates, by interactions between protein molecules, and by oxidation. These reactions are promoted by the temperature

and duration of the heat treatment used. The central process is the combination of free amino groups with other reactive groups to form enzyme-resistant inter- and intra-molecular bonds.

The nutritional consequences of these reactions are reduced solubility and digestibility of the protein and a prolonged digestion. Loss of palatability and destruction of several amino acids by oxidation may also lead to a reduction of nutritive value of the heat-damaged protein feed.

Inadequate methods in monitoring nutritive value can lead to false conclusions. *In vivo* bioassays provide information on possible nutritional damage to proteins, though they reveal changes in protein utilization only when the limiting amino acids are affected. From the practical point of view, the content of reactive lysine with free epsilon-amino group may be a useful indicator of heat damage in protein feeds having a surplus in lysine.

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OPTIMIZING PROTEIN QUALITY OF MIXTURES OF BLOOD MEAL, FEATHER MEAL AND BONE MEAL

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The protein quality of two- or three-component mixtures of blood meal, feather meal and bone meal was characterized by amino acid scores and rat net protein utilization (NPU) values.

A graphic method designed to find optimum levels of the limiting essential amino acids in the mixtures was suitable for predicting the optimum of NPU values determined by feeding rats with diets having 10% crude protein.

The protein quality of mixtures of blood meal, feather meal and bone meal showed an optimum if blood meal constituted 60% of the protein content of the mixtures; however, poor feed intake and growth data were obtained.

Keywords: Blood meal, feather meal, bone meal, protein quality, amino acid score, net protein utilization, mixtures of proteins.

Abattoir by-product meals of poor quality, such as blood meal, feather meal and bone meal do not support the growth of young rats when fed as sole protein source in diets with protein levels allowing maximum efficiency of utilization. These by-product meals have specific essential amino acid deficiencies, though they also have relative excesses (Hegedűs et al., 1983*a*).

The amino acid profile of these poor-quality abattoir by-product meals can be corrected by enrichment with synthetic amino acids. However, this approach does not consider the relative excesses of other essential amino acids. The overall amino acid pattern of the protein can also be improved by combining blood meal, feather meal and bone meal in order to balance each other's deficiencies and excesses.

To find optimum mixtures of abattoir by-product meals, various amino acid scores can be used (Hidvégi and Békés, 1985) for predicting maximum utilization of protein; besides, calculation of the relatively simple chemical score is also of informative value.

The present work was performed to study the protein quality of three-component mixtures of blood meal, feather meal and bone meal in rat experiments (NPU value) and to predict the optimum utilization of the mixtures by chemical scores using a rapid graphic method.

Materials and methods

Origin of samples

The animal protein meals examined were produced in a dry rendering plant on an industrial scale by heating (130 °C, 30 min) and drying. Blood meal was produced from slaughterhouse flow-off blood in a batch rendering cooker. The raw material of feather meal consisted of wet feathers, a by-product of industrial poultry slaughterhouses, which was heat-processed by steam pressure without any chemical treatment. Bone meal was produced from industrial raw bone.

Methods

Raw chemical composition of the samples (dry matter, crude protein, ether extract, ash, pepsin digestibility) was determined according to the Hungarian standard MSZ 6830 (1981).

Amino acid analyses were performed by ion-exchange column chromatography according to the method of Mason et al. (1980), using a Durrum D-500 type amino acid analyser. Tryptophan was determined by the method of Matheson (1974).

The amino acid score, i. e. *chemical score*, was calculated from the amino acid composition. The concentration of each essential amino acid (g/16 g N) was expressed in per cent of the requirement of the same amino acid for the growing rat (NRC, 1978). The amino acid showing the lowest proportion is called the limiting amino acid and its relative concentration is the chemical score, i.e. amino acid score.

Net protein utilization (NPU) expresses the retained part of the nitrogen intake. It was determined by a slaughter method as described in a previous work (Hegedűs et al., 1981). Ten-day feeding experiments were carried out in LATI CFY, SPF, weaned male rats, kept in wire cages, six rats in each cage. The composition of the test diets fed *ad libitum* was: cooking fat, 14.25% (w/w); sunflower oil, 0.75% (w/w); non-nutritive cellulose powder (Macherey Nagel, MN 300), 5% (w/w); glucose 15% (w/w); vitamin and mineral premix, adequate to meet the rats' requirements (LATI, Budapest), 3% (w/w); protein source to give 10% crude protein in the test diet, and maize starch to give 100% (w/w). The protein-free control diet was the same, except that it contained maize starch instead of the protein source. After 10 days' feeding, feed intakes and body weights were measured. NPU was calculated by a regression equation between NPU and net protein ratio (NPR). NPR was obtained by dividing the weight difference of the test and control groups by the nitrogen intake of the test group.

Results and Discussion

Optimizing the levels of limiting amino acids by a graphic method

The chemical composition and amino acid content of blood meal, feather meal and bone meal, as summarized in Table I, showed characteristic differences. Blood meal and feather meal contained high levels of crude protein, whereas bone meal had high ash content. Blood meal had a surplus in lysine and leucine, whereas feather meal was deficient in lysine, but rich in cystine. Bone meal contained low levels of tryptophan and cystine, due to its high collagen content.

A better amino acid pattern can be achieved by mixing these ingredients to minimize deficiencies and cut down excesses of essential amino acids. To find optimum mixing ratios, a simple graphic method was used, which is illustrated in Fig. 1.

Figure 1 was prepared by plotting relative concentrations of individual amino acids of the pure ingredients on the right and left ordinates. The two points for the same amino acids were then connected with a straight line, for mixing — in this case — has an additive character. The lowest line shows the relative concentrations of limiting amino acids. The point of intersection,

Table I

Chemical composition of the ingredients used in the mixtures

	Blood	Feather meal	Bone
Dry matter (g/100 g)	90.0	91.8	97.6
Crude protein (g/100 g)	80.5	80.3	24.9
Amino acid N (g/100 g N)	77.0	82.0	74.2
Pepsin digestibility (%)	85.0	81.0	79.5
Ether extract (g/100 g)	4.1	6.3	10.3
Ash (g/100 g)	4.3	5.2	59.2
Essential amino acids (g/16 g N)			
Lysine (LYS)	9.6	1.7	4.2
Methionine (MET)	1.4	0.6	0.7
Cystine (CYS)	1.2	4.0	0.1
Tryptophan (TRP)	1.3	0.7	0.2
Threonine (THR)	4.5	3.9	2.0
Leucine (LEU)	11.8	7.7	3.5
Isoleucine (ILE)	1.3	4.6	1.5
Valine (VAL)	8.0	7.3	2.9
Phenylalanine (PHE)	5.0	4.4	2.2
Tyrosine (TYR)	4.1	2.1	1.0

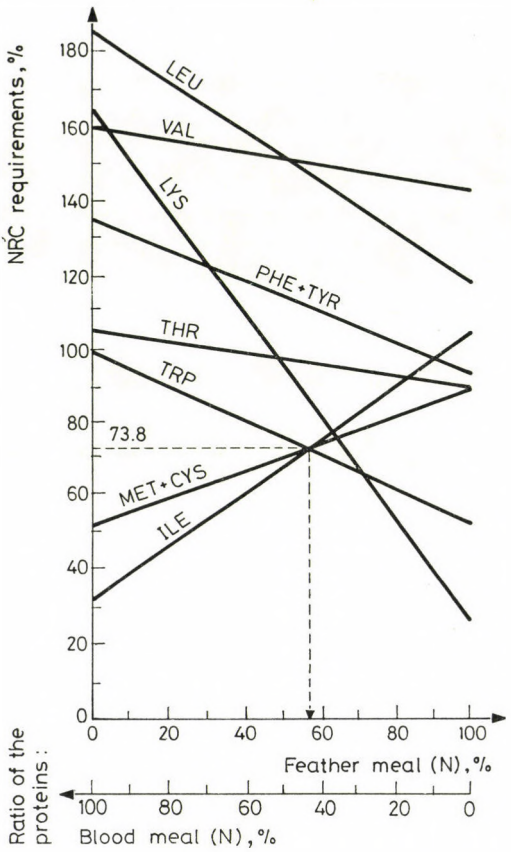

Fig. 1. Illustration of the graphic method for determination of the optimal complementary mixture of blood meal and feather meal

* Percentage of NRC requirements (1978) for the growing rat. Optimum of amino acid scores: 73.8 Ratio of the proteins: Feather N/Blood N=57/43. Ratio the ingredients (w/w): Feather meal/Blood meal=57.1/42.9.

i.e. that having the highest value of limiting essential amino acids, gives the optimal mixture, which is marked by dotted line in Fig. 1.

As shown in Fig. 1, lysine and isoleucine are the limiting essential amino acids for feather meal and blood meal, respectively. Note that tryptophan can be limiting when the two protein sources are mixed. The graphic method for optimizing levels of limiting essential amino acids in mixtures of protein sources also supplies information about the essential amino acid profile (deficiences and excesses) of all the mixing ratios. The graph can be simplified to include only the three or four essential amino acids which are limiting most frequently (e.g. lysine, methionine, tryptophan, threonine).

This graphic method is also useful to calculate an individual essential amino acid requirement supplied by a given mixing ratio in per cent. Criteria

other than chemical score may be used for monitoring the effect of mixing ratios if they are supposed to have an additive nature (e.g. more sophisticated amino acid indices, digestibility, etc.).

Optimum levels of limiting essential amino acids in blood meal, feather meal and bone meal as well as those of their two- and three-component mixtures are summarized in Table II. The highest, amino acid score (73.8) was achieved when blood meal and feather meal were mixed. All of the amino acid scores of the optimized two-component mixtures were higher than those of the pure components.

The optimum amino acid score of the three-component mixtures of blood meal, feather meal and bone meal was 63.0. The optimum mixing ratios (%, w/w) of the ingredients to achieve optimum amino acid scores are shown in Table III.

Prediction of protein quality of mixtures by amino acid scores is a relatively simple and fast method. However, there are certain factors, unrelated to the amino acid content of the diet, which can influence the utilization of

Table II

Optimized levels of limiting amino acids in blood meal, feather meal and bone meal and their mixtures

Protein source	Limiting amino acid	Amino acid score[a]
Ingredients		
Blood meal	ILE	30.9
Feather meal	LYS	29.3
Bone meal	TRP	15.4
Two-component mixtures:		
Blood/Feather	ILE/TRP	73.8
Blood/Bone	ILE/MET + CYS	32.8
Feather/Bone	LYS/TRP	42.2
Three-component mixtures:		
Bone/Feather/Bone	LYS/MET + CYS	63.0

[a] Concentration of limiting essential amino acid (g/16 g N) expressed in per cent of the requirement of the same amino acid for the growing rat (NRC, 1978)

Table III

Optimum mixtures of blood meal, feather meal and bone meal

Maximum of the amino acid score	Ratio of the ingredients in the mixture (%, w/w)		
	Blood meal	Feather meal	Bone meal
73.8	42.9	57.1	—
32.8	21.5	—	78.5
42.2	—	40.8	59.2
63.0	28.7	29.8	41.5

dietary proteins (Kofrányi, 1967; Bressani, 1977; Woodham, 1978; Hegedűs et al., 1983*b*). To check the practical value of the graphic optimizing method, rat feeding tests were also carried out.

Protein quality of mixtures of blood meal, feather meal and bone meal in rat feeding experiments

Mixtures of feather meal and bone meal. The rat performance data used to obtain NPU values are summarized in Table IV. In the mixtures feather meal was substituted for bone meal on a nitrogen basis in proportions of 10, 20, 30, 40, 50, 60, 70, 80 and 90%. Initial and final body weights as well as NPU values are shown in Fig. 3.

Increasing substitution of the nitrogen of feather meal for nitrogen of bone meal resulted in a complementary effect as indicated by the NPU value. Amino acid scores showed the same complementary effect as illustrated in Fig. 2. The ratio of proteins at the optimum of the NPU values as well as chemical scores was practically the same: 70% feather-N/30% bone-N in the

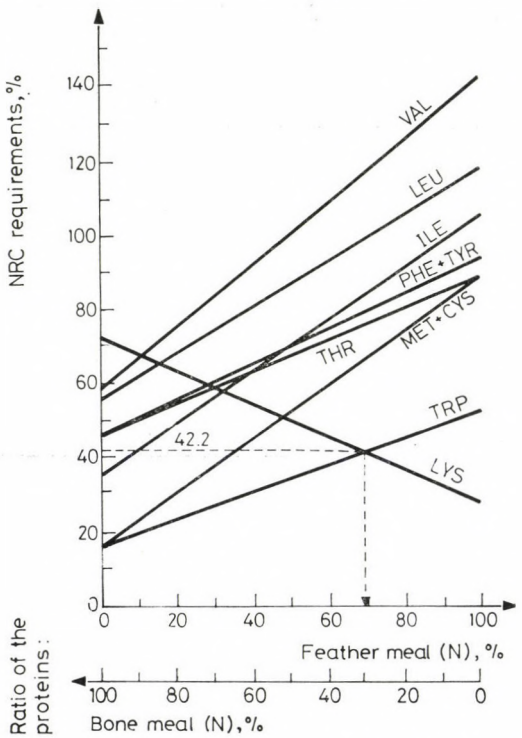

Fig. 2. Amino acid scores of mixtures of feather meal and bone meal
* Percentage of NRC requirements (1978) for the growing rat. Optimum of amino acid scores: 42.2 Ratio of the proteins: Feather N/Blood N=69/31. Ratio of the ingredients (w/w): Feather meal/Blood meal=40.8/59.2

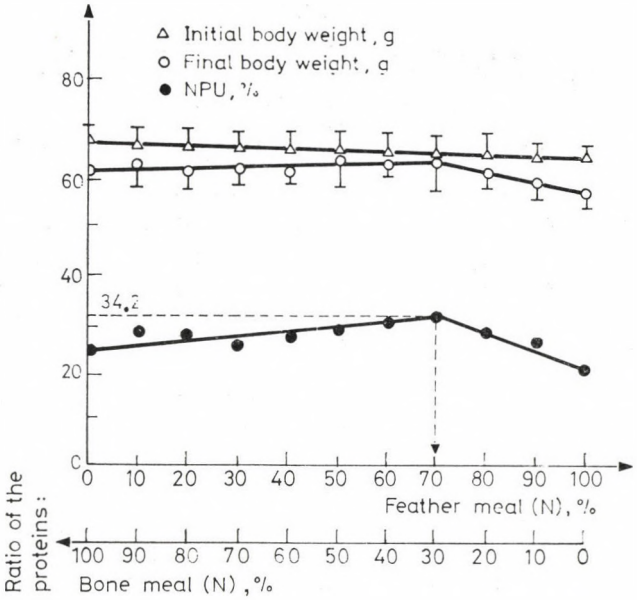

Fig. 3. Protein quality of mixtures of feather meal and bone meal

Table IV

Net protein utilization of mixtures of feather meal and bone meal in 10-day rat feeding test

Protein source[1]	Feed intake[2] (g/rat/10 days) (I)	Initial body weight (g/rat)	Final body weight (g/rat) (W)	NPU[3] (%)
100% FM	45.57	66.58 ± 2.25	59.50 ± 2.85 a[4]	23.3
90% FM + 10% BM	52.67	66.58 ± 2.46	62.00 ± 3.68 b	29.2
80% FM + 20% BM	56.20	66.58 ± 3.68	63.08 ± 2.78 b	30.8
70% FM + 30% BM	64.91	66.58 ± 3.50	65.67 ± 5.72 b	34.2
60% FM + 40% BM	61.85	66.58 ± 3.06	64.42 ± 2.49 b	32.1
50% FM + 50% BM	67.66	66.58 ± 3.69	64.83 ± 4.99 b	30.5
40% FM + 60% BM	57.81	66.58 ± 3.37	62.58 ± 1.66 b	28.4
30% FM + 70% BM	56.08	66.58 ± 3.41	62.83 ± 2.73 b	26.0
20% FM + 80% BM	52.05	66.58 ± 3.25	61.75 ± 2.62 b	28.5
10% FM + 90% BM	60.23	66.58 ± 3.09	63.00 ± 4.09 b	28.6
100% BM	52.88	66.58 ± 2.19	60.58 ± 1.50 a	23.6
Protein-free control	53.66	66.58 ± 3.12	53.83 ± 3.24 (W_k)	—

[1] % of nitrogen provided by each source. FM = feather meal, BM = bone meal;
[2] Average feed intake of six rats. Individual feed intake was not measured;
[3] Net protein utilization was calculated from net protein ratio (NPR). NPU = 3 NPR.

$$NPR = \frac{W - W_k}{0.016\ I};$$

[4] Body weights with different letters deviate significantly ($P < 0.05$)

Table V

Net protein utilization of mixtures of blood meal, feather meal and bone meal in 10-day rat feeding test

Protein source[1]	Feed intake[2] (g/rat/10 days) (I)	Initial body weight (g/rat)	Final body weight (g/rat)	NPU[3] (%)
100% Blood meal (BLM)	42.72	63.83 ± 3.82	55.67 ± 4.99 a[4]	8.8
10% BLM + 85% FM + 5% BM	56.22	63.83 ± 3.75	57.58 ± 5.82 b	13.0
20% BLM + 75% FM + 5% BM	40.63	63.83 ± 4.84	58.00 ± 5.24 b	20.0
30% BLM + 65% FM + 5% BM	51.56	63.83 ± 4.40	59.75 ± 3.90 b	22.2
40% BLM + 55% FM + 5% BM	47.17	63.83 ± 4.86	59.58 ± 7.30 b	23.6
50% BLM + 45% FM + 5% BM	52.19	63.83 ± 4.72	60.66 ± 2.94 b	25.0
60% BLM + 35% FM + 5% BM	54.65	63.83 ± 3.66	63.00 ± 4.48 b	32.2
70% BLM + 25% FM + 5% BM	52.96	63.83 ± 3.33	62.00 ± 8.74 b	29.5
80% BLM + 15% FM + 5% BM	38.66	63.83 ± 4.25	58.00 ± 9.75 b	21.0
10% BLM + 80% FM + 10% BM	52.57	63.83 ± 2.93	59.92 ± 3.93 b	22.3
20% BLM + 70% FM + 10% BM	61.77	63.83 ± 3.86	61.25 ± 4.69 b	23.0
30% BLM + 60% FM + 10% BM	61.22	63.83 ± 4.02	61.83 ± 7.99 b	25.0
40% BLM + 50% FM + 10% BM	57.19	63.83 ± 4.12	61.33 ± 6.82 b	25.0
50% BLM + 40% FM + 10% BM	61.54	63.83 ± 3.43	63.58 ± 4.86 b	30.3
60% BLM + 30% FM + 10% BM	59.52	63.83 ± 4.11	63.67 ± 6.34 b	31.6
70% BLM + 20% FM + 10% BM	50.12	63.83 ± 1.94	59.42 ± 5.78 b	21.6
80% BLM + 10% FM + 10% BM	50.18	63.67 ± 3.56	57.42 ± 5.40 b	14.1
Protein-free control	56.43	63.83 ± 3.24	53.67 ± 2.93 (W_k)	—

[1], [2], [3], [4]: see Table IV;
BLM: blood meal; FM: feather meal; BM: bone meal

rat experiment, and 69% feather-N/31% bone-N according to the graphic optimization.

The protein quality of the optimum mixture of feather meal and bone meal did not allow weight gain of young growing rats given 10 per cent raw protein in the diet; however, the efficiency of protein utilization could be improved.

Mixtures of blood meal, feather meal and bone meal. Feather meal and blood meal showed a striking complementary effect as illustrated in Fig. 1. Therefore, mixtures were made for gradual substitution of feather meal for blood meal. To obtain three-component mixtures 5% and 10% bone protein was involved, for the use of a higher proportion of bone meal in the diets is of no practical interest.

The rat performance data used to obtain NPU values are summarized in Table V. Initial and final body weights as well as NPU values of the mixtures are shown in Figs 4 and 5.

Optimum utilization of ingested protein was obtained if blood meal constituted about 60% of the protein of the mixtures. The use of 5% or 10%

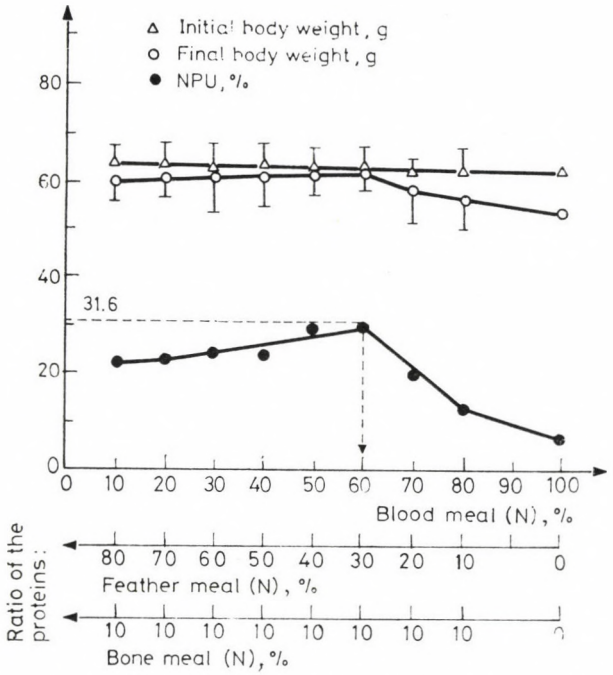

Fig. 4. **Protein quality of mixtures of blood meal and feather meal supplemented with 5% bone meal N**

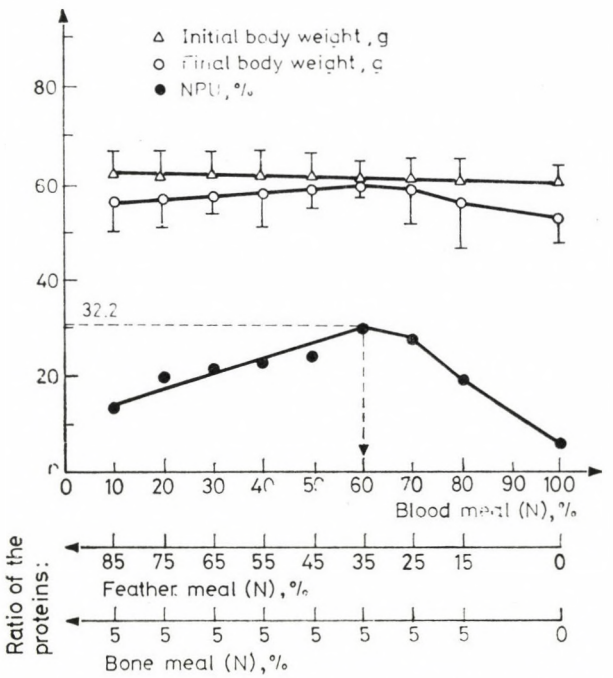

Fig. 5. **Protein quality of mixtures of blood meal and feather meal supplemented with 10% bone meal N**

bone meal N did not alter significantly the NPU value of the optimum mixture and the final body weights of the rat groups.

The optimum mixtures of blood meal and feather meal either with 5% or 10% bone N were inadequate to promote weight gain of young rats kept on diets containing 10% protein.

The relatively low feed intake data also show that this type of mixtures cannot be used efficiently as sole protein source. However, the efficiency of protein utilization can be improved by mixing them in proper ratios.

Sixty % blood meal protein in three-component mixtures of blood meal, feather meal and bone meal ensures moreover a high lysine content (see Fig. 1) which exceeds the requirement of the rat. Therefore, such optimized mixtures of blood meal, feather meal and bone meal can be advantageously used to complement cereal-based diets.

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EVALUATION OF THE ENERGY VALUE OF RABBIT FEEDS: A CRITICAL REVIEW

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An analysis is given of the methods actually used for the determination and calculation of the energy value of rabbit feeds and feedstuffs. The apparent digestible energy (DE) system is recommended. Standardization of the applied methodology for *in vivo* determination is emphasized. Four to six *ad libitum* fed growing rabbits are necessary to obtain sufficiently accurate results. The reliability of the estimation of the feeds' energy content, based on the table values of the ingredients, is discussed. The importance of using recently and properly determined data is stressed. Multiple regression equations, based on the results of the chemical analysis of feeds, explain 85–90% of the variability of DE content. The first attempts with *in vitro* techniques are encouraging.

Keywords: Energy value, determination methods, rabbit feed, critical review.

The growing rabbit is able to adjust its voluntary dry matter intake to the energy density of its feed mixture (Lebas, 1975; Bombeke et al., 1978; Spreadbury and Davidson, 1978; Dehalle, 1981; Fekete and Gippert, 1985). This regulation of feed consumption, aimed at achieving a constant daily apparent digestible energy (aDE) intake, is possible between energy concentration limits of 9.2 to 13.4 MJ aDE/kg air-dry matter (Partridge, 1986). Owing to this, the recommended nutrient concentrations should be related to the dietary energy level. For this reason, the energy content of the feed is the basis of ration formulation. The energy value is also a very important item for the breeder to judge both the quality and the price of feeds. A 0.5 MJ/kg increase or decrease of the DE content would be associated with a fall or rise of the feed conversion ratio by nearly 0.2 (Lebas et al., 1982; Maertens and De Groote, 1987). Furthermore, the importance of the energy/protein ratio has clearly been demonstrated by De Blas et al. (1984), Dehalle (1981) and Fekete and Gippert (1985).

For evaluating the energy value of rabbit feedstuffs and feed mixtures, data obtained for other animals (mostly swine) have often been used. Nevertheless, several studies have clearly indicated that the digestibility of the ration by rabbits considerably differs from that by other species (Nehring et al., 1963; Ingals and Thomas, 1964). A correct calculation of the dietary energy

concentration is therefore possible only if data derived from rabbit experiments are used. In recent years considerable research has been conducted all over the world (Lebas and Colin, 1976; Parigi-Bini and Dalle-Rive, 1977; Colin and Lebas, 1976; Fekete and Papp, 1981; Gippert et al., 1981; Fekete and Gippert, 1981, 1983, 1986; Balogun, 1984; Battaglini and Grandi, 1984; INRA, 1984; De Blas et al., 1984; Maertens and De Groote, 1984, 1987; Maertens et al., 1986, 1987, 1988; Fekete, 1985; Bokori, 1986; Corino, 1987; Fekete et al., 1988; Vetési, 1990) to obtain a useful energy evaluation system for rabbits. However, no generally accepted and used method for evaluating and predicting the energy value of feeds has become available. For this reason, a round-table discussion was organized within the frame of the 4th World Rabbit Congress (Budapest, 10–14 October 1988). The purpose of this article is a critical overview of this subject, supplemented with the comments of the round-table discussion.

The choice of feed energy systems for rabbits

Energy losses by the urine are small and for balanced feed mixtures rather similar: 4–6% of the DE (Parigi-Bini and Cesselli, 1976; Fekete and Papp, 1981). Since there is a high correlation between the digestible and the metabolizable energy (ME), there is no particular advantage in using the latter.

There is no doubt that the net energy (NE) would be the most precise estimate of the feeds' energy value because it represents the proportion of the gross energy utilized by the animal for maintenance and production. Therefore, the net energy concentration depends upon the "goal" of utilization (Fekete, 1988). Furthermore, the determination of the NE by means of respiration trials and/or comparative slaughter experiments is rather difficult. Using regression equations, it is also possible to calculate the NE content based on the digestible composition. Evidences that a calculated NE value would be more accurate than evaluation of the digestible or metabolizable energy do not exist.

For these reasons, the net energy system remains a future aim and the digestible energy values are actually the most commonly used data in rabbit nutrition. The relatively easy determination and the high correlation with the animal's production are extra arguments. Not all of the energy losses in the faeces originate directly from the actually consumed feed. It is difficult to determine this endogenous fraction for any animal species, but especially for the rabbit, owing to the phenomenon of caecotrophy (Fekete and Bokori, 1985). Therefore, caecotrophy should be considered during the standardization of digestibility trials.

In vivo determination of the DE value

Based on the work done at INRA (Lebas and Colin, 1976; Colin and Lebas, 1976), usually the following general scheme is applied:

— rabbits are housed individually in digestibility (metabolic) cages;
— after a preliminary (adaptation) period of 5–10 days, a balance trial of 7 days (or 2×4 days) is performed;
— the daily total amount of faeces is collected.

Depending on the laboratory, growing or adult rabbits are used. Arguments for growers are that their feed represents the main part of commercial rabbit feed mixtures and that after 9 weeks of age digestibility is relatively constant (Lebas, 1973; Maertens and De Groote, 1982; Fekete and Lebas, 1983; Hullár and Gippert, 1986). For adult rabbits, the measured digestibility coefficients depend much more on the feeding level and on the biological status (maintenance, pregnancy, lactation) of the animals (Lebas, 1979; Maertens and De Groote, 1982; Hullár and Szabóné-Lacza, 1988).

There undoubtedly exist differences between breeds, but the variance between the commercially used strains (New Zealand White, Californian or the meat crossings) is limited (Lebas, 1973; Maertens and De Groote, 1982; Hullár and Gippert, 1986). Differences between males and females are also negligible during the growing period (Colin and Lebas, 1976; Maertens and De Groote, 1982; Fekete and Bokori, 1986; Hullár and Gippert, 1986).

As it is emphasized by many researchers, there is a considerable individual variation in the efficiency of digestion (Proto, 1964; Lebas and Colin, 1976; Gioffre et al., 1985) and even the influence of the origin (litter) is not negligible (Maertens and De Groote, 1982). At least four, or rather six, replicates per treatment are necessary to obtain digestible energy values with sufficiently little variation (Maertens et al., 1986), even if a normal growth rate and feed intake is supposed.

Feed restriction (rationing) has a significant influence on the digestibility of feeds (Kalugin, 1980; Fekete and Gippert, 1981; Ledin, 1984; Xiccato and Cinetto, 1988). For this reason the results of digestibility trials with growers are comparable only if the animals are fed *ad libitum*.

Markers are very seldom used for rabbit digestibility trials because the excreted faeces is little in amount.

Consequently, total faeces collection was chosen. The dry matter content of rabbit faeces is high in comparison to other species (poultry, cattle). Losses during air-oven drying at 60–70 °C are negligible, but a preliminary heating for 1 h at 105 °C or freeze-drying leads to more accurate results.

Fistulated rabbits (Gidenne and Bouyssou, 1987) or partial digestibility is of high interest rather for the measurement of nitrogen metabolism but not

for energy evaluation. Caecotrophy is a physiological phenomenon in the rabbit; any attempt to prevent it or the separation of the caecotroph from the hard faeces leads to modification of the digestibility coefficients (Fekete and Bokori, 1985).

The energy evaluation of raw materials

Till now, the most commonly used method has been the one-level assay. A certain amount of the basal diet is substituted for by the experimental feedstuff. The basal and experimental diet are measured in a two-step digestibility trial. Using the "difference principle" (Schürch, 1969), the value of the tested feedstuff can be calculated. For most feedstuffs the inclusion level is between 20 and 60% (Lebas and Cheriet, 1981; Fekete and Gippert, 1983, 1986; Balogun, 1984; Beltran et al., 1984; Maertens and De Groote, 1984). When the feedstuff is palatable and its composition is not too far from the rabbit's nutrient requirements, it is even possible to feed the same feedstuff throughout the digestibility trial (Duchenne, 1980; Maertens and De Groote, 1981). It is also possible to substitute the test feedstuff for a component of known digestibility (first of all starch).

The one-level inclusion (incorporation) method is criticised as follows.

a) The error of the DE value of a feedstuff decreases parallel with the increasing substitution level. On the other hand, the high inclusion levels are far above the practically used ones.

b) For a variety of reasons (palatability, physicochemical properties, etc.) some feedstuffs (e.g. fats, molasses, feather meal, etc.) cannot be incorporated in a percentage above 20%.

c) The "additivity principle" (Kladowshtschikow and Samkow, 1975), suggesting that there is no interaction between the basal diet and the tested feedstuff, may not be valid in the practice.

On the basis of these arguments, when an interaction can be expected between the nutrients (e.g. high fat content) or the inclusion level is limited, the multi-level assay is the method of choice to evaluate feedstuffs. This technique means testing of the feedstuff at several levels of incorporation, coupled with a regression analysis to calculate the digestible energy value. At a given level of inclusion the DE concentration of a feedstuff is determined by extrapolating the actual values, using the DE content of the basal diet as reference (Wiseman, 1984). Since for evaluating the digestibility of a feedstuff at least 2, but rather 4, inclusion levels seem recommendable, the multi-level technique is time consuming and expensive, and it should be reserved for the above-mentioned special raw materials. There are no experimental evidences that the additivity principle should be rejected for the ordinary ("normal") rabbit feedstuffs like alfalfa meal (Maertens and De Groote, 1981). The nature

of the basal diet also seems to be important in determining the feedstuff's DE value. Sufficient experimental data have not yet been obtained on how to choose or formulate the basal diet according to the tested feedstuff.

Calculation of the DE content of rabbit feeds

I. *On the basis of the digestible composition*

Multiple linear regression equations have been developed, based on the digestible composition, to calculate the (apparent) digestible energy content of feedstuffs and feed mixtures (Nehring et al., 1963; Maertens et al., 1987). If the chemical composition and the standardized (tabulated) digestibility coefficients of nutrients are available, the DE content can be calculated with high accuracy. The deviation from the directly determined value is only 1–2% (Fekete and Papp, 1981; Maertens and De Groote, 1981).

II. *On the basis of the tabulated, individual DE values of feedstuffs*

The most widely applied method for estimating the digestible energy concentration of a feed mixture is summing up of the DE values of the ingredients, taking into account their percentage. A lot of tables are available (Van Schoubroeck and Cloet, 1968; NRC, 1977; INRA, 1984; Schlolaut, 1984; Finzi and Gualteria, 1986). The data in the summarizing tables are mainly based on experiments carried out more than 20 years ago. With respect to the change of the feeding methods, the insufficiency of the number of animals used or, sometimes, the diversity of the methodology, the usefulness of the above-mentioned tables for calculation of the DE concentration can be queried. For these reasons, efforts have been made in several countries to obtain more reliable values, especially for the actually used raw materials. The following experimental data have been published recently: Fekete and Gippert (1986), Raharjo et al. (1986) and Maertens et al. (1987). The majority of these data are included in the book of Cheeke (1987). Some limited data are available in the French, Italian and Spanish literature (e.g. Pascual and Carmona, 1980).

The problems implicated in employing this method are the following.

— The aDE content of feedstuffs has been found highly variable by us. The reasons for the variability could be sought in the methodology, in the divergent composition of some feedstuffs (mainly roughages, e.g. alfalfa meal) and in using non-average batch. Therefore, since it seems that each table bears the "stamp" of the given laboratory, for the practice it is better to use one table and not to mix the data of the different tables. In the latter case energy evaluation would be incorrect. Some feedstuffs would be systematically over- or underestimated.

— Using the table values to calculate the DE content, one may assume that interactions between the ingredients do not occur, and the digestible energy value is not affected by the inclusion level. Recent results, mainly obtained for poultry, have clearly demonstrated that in the case of a high fat supplementation the described assumption may not agree with the reality. Unsaturated fats improve the digestion and the absorption of the added saturated fat. Even the transit time in the digestive tract can be affected and the digestion of other nutrients improves. This phenomenon is known as synergism (De Groote et al., 1987). Recently interactions between the fibre components and other nutrients have been demonstrated in rabbits (Gidenne, 1987; Gidenne et al., 1987).

Furthermore, sufficient experimental evidence exists indicating that the incorporation level of fats influences their energy value. This effect has also been demonstrated in rabbits (Maertens et al., 1986). Using the data of the multi-level assay and calculating with energy values belonging to the given incorporation level lead to a more accurate energy evaluation.

The adjustment of the feedstuffs' composition (mainly in the computer matrix) is also necessary, for the tabulated composition may not agree with the actual chemical composition. Two methods are available to correct the data according to the applied quality (Maertens et al., 1988).

a) Making use of the average digestibility coefficients of the feedstuff concerned and a regression equation(s) based on the digestible composition.

b) Using the corresponding regression equation(s) for each group of feedstuffs based on the data of the actual chemical analysis, e.g. in the case of alfalfa meal:

$$\text{aDE, MJ/kg DM} = 8.47 + 0.237\ \text{CP} - 0.148\ \text{CF},$$

where aDE is the apparent digestible energy, DM means the dry matter, CP and CF the crude protein and crude fibre content, respectively, in percentage.

It has to be emphasized that the calculation of the digestible energy value, based on the tabulated data, is possible only if the dietary ingredient composition is known (open formulation).

Prediction of the DE content by means of chemical analysis

Efforts have been made for all species to predict the DE content when the composition of the ingredients is unknown. Reliable relationships (multiple regression equations) between certain chemical parameters and the digestible energy concentration are being searched for. The applicability of these equations needs high accuracy (characterized by the F value, R^2 and rsd), requirement of few chemical parameters, low cost, good reproducibility of the chemical analyses used and lack of significant correlations between the independent

variables (in the reality certain chemical characteristics can highly correlate, but this should not be necessarily true for another feed or feedstuff).

Numerous authors stress the negative relationship between the fibre level (Weende or Van Soest) and the energy content (Jentsch et al., 1963; Parigi-Bini and Dalle-Rive, 1977; Battaglini and Grandi, 1984; De Blas et al., 1984; Corino, 1987). This is self-evident because in rabbits fibre digestibility is low. At the same time, none of these relationships explains more than 75% of the observed variation in the DE content. When a diet with high fibre digestibility (e.g. sugar beet pulp) is included in the pool of elaboration, the accuracy is even worse (De Blas et al., 1984; Maertens et al., 1988). The equations, based on the acid detergent fibre (ADF) level, did not give better estimation than the use of crude fibre (CF) content (Maertens et al., 1988). This is not surprising, for there is a strong correlation between the ADF and CF levels (Pagono-Toscano et al., 1986; Maertens et al., 1988).

Depending on the authors, the highest R reached was 0.85, using the fibre components and the gross energy as independent variables (De Blas et al., 1984; Battaglini and Grandi, 1984; Corino, 1987; Maertens et al., 1988). However, direct determination of the gross energy is possible only in a limited number of laboratories. Other regressions with two parameters (e.g. CF and ash) were also suggested (Fekete and Gippert, 1986; Maertens et al., 1988), without further improvement in accuracy.

Equations with 3 or more chemical data explain already 90% of the observed variation in aDE content (Maertens et al., 1988). This is still lower than optimum. Data from different laboratories have to be used together or other chemical parameters should be taken into account to increase the accuracy of estimation. The use of a more detailed analysis of the fibre fraction as proposed by Theander and Aman (1981) has to be considered.

In vitro techniques for estimating the energy content

Limited research work is available concerning the energetic evaluation of rabbit diets by *in vitro* techniques which simulate the digestive processes of the organism. At the same time, enzymatic methods were recently investigated in pigs and cattle. The results are encouraging, especially those which were obtained by the cellulase method (De Boever et al., 1986). Future research is necessary to evaluate the suitability of these methods as predictors of the energy content, exclusively of rabbit feeds and feed mixtures.

Another *in vitro* technique, well known as used in ruminants, uses rumen fluid to simulate the digestive processes in the forestomach (Tilley and Terry, 1963). Recently, its modified version for rabbit diets has been published (Scholtyssek and Seim, 1988). Using data on the gas production of caecal bacteria and the protein and fibre level of the ration, multiple regression equa-

tions have been established for prediction of the DE content. The calculations fit more than 90% of the variation in aDE concentration, but the number of tested diets was limited (8). Consequently, the usefulness and accuracy of this method are yet to be confirmed.

Conclusions

For more than 10 years "joule" has been internationally recognized as the single official energy unit. Efforts have to be made to really replace calory in both research and practice.

The available DE values are influenced by several factors, the most important being composition of the feed or feed mixture, individual variability, feeding level (i.e. the amount of the ration) and physiological status. Taking into account that evaluation is always only an approximation to a very variable value, the methodological questions bear fundamental importance. The accuracy of the estimation is extremely important to satisfy the animals' nutrient requirements, to formulate least-cost diets and to interpret the results of the nutrition experiments.

Although during the last years striking progress has been made in the energetic evaluation of rabbit feedstuffs and feed mixtures, further research is necessary in different fields. The accuracy of the evaluation of raw material needs to be improved. Each modifying factor (lactation, fat and/or oil supplementation, mycotoxins, etc.) influencing digestibility and the real relationships between the chemical data and DE content should be clarified. Also, further development of the *in vitro* techniques and collection of basal data are necessary for a future net energy system.

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THE EFFECT OF ANIMAL FAT AND VEGETABLE OIL SUPPLEMENTATION OF FEEDS OF DIFFERENT ENERGY CONCENTRATION UPON THE DIGESTIBILITY OF NUTRIENTS AND SOME BLOOD PARAMETERS IN RABBITS

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The effect of mixed animal fat and sunflower oil supplementation (5%) of a feed of medium (12.02 MJ DE/kg) and low (8.54 MJ DE/kg) energy concentration upon the digestibility of nutrients and on some blood parameters was investigated. The ether-extractable content of feed and faecal samples was determined by diethyl ether extraction (after Soxhlet) and the total (true) fat level was measured by the method of Stoldt (1952), *viz.* petroleum ether extraction of samples pretreated with 4 N HCl.

In the majority of cases the voluntary feed intake decreased after the addition of animal fat or vegetable oil. Mixed animal fat supplementation significantly (by 5 and 11 units) improved the digestibility of the ether extract if added to either of the basal diets. The digestibility of crude fibre and N-free extract increased only in the case of the basal diet of low energy concentration (by 4 and 7 units, respectively). Sunflower oil addition produced changes of the same tendency (9, 9, 28 and 5 units). The digestibility of the crude protein was practically not altered by either supplementation.

The total fat content of feed and faecal samples proved to be higher than that of the ether extract, on an average by 27 and 100%, respectively. Consequently, the digestibility coefficients of the total fat are by 10 units lower than those of the ether extract. The DE concentrations, calculated by means of the digestible ether extract or the total fat content, differed significantly only between the basal diets.

The addition of mixed animal fat to either of the basal diets increased the blood concentration of total lipids (by 18 and 32%, respectively), while that of sunflower oil did not cause such an alteration. Neither fat nor oil supplementation had an effect on the cholesterol level of the blood plasma. Both the animal fat and the vegetable oil surplus modified the fatty acid composition of blood lipids. It was characteristic of each treatment that the proportion of polyunsaturated fatty acids increased.

Keywords: Animal fat and vegetable oil supplementation, rabbit feed, digestibility, ether extract *vs.* total fat, blood parameters.

Fat enrichment of feed mixtures is a well-known and, especially in poultry nutrition, widely practised method. Its advantages include that, parallel with the reduction of the feed intake, it enhances the growth rate of young animals (i.e. improves the feed conversion ratio). The added fat is a source of essential fatty acids, facilitates the absorption of fat-soluble vitamins and, through the reduction of powder formation, improves palatability and pellet building (Kakuk, 1981; Cheeke, 1987). If the appropriate energy/protein (more exactly: energy/amino acid) ratio is taken into consideration, the quality of the slaughtered product (carcass) was not worsened by the supplementation (Patrick and Schaible, 1981).

The picture of the effect of fat supplementation upon the digestibility of nutrients is not so uniform (Lang, 1981). According to the review of Schürch (1969) the fat content of the feed very rarely modifies the digestibility of nutrients, and if it does, above all in ruminants, it acts by means of the rumen microorganisms. The digestibility of dry matter, ether extract and crude protein did not change after the addition of vegetable oil to the feed (Thacker, 1956). Similarly, tallow supplementation did not modify the digestibility of dry matter, crude protein, crude fibre and gross energy (Parigi-Bini et al., 1974).

Lebas (1975*a*) reports that the addition of corn oil to the diet decreased the digestibility of dry matter, organic matter and energy. In the experiment of Teleki and Darwish (1969) an increase of the fat concentration from 2.68 to 5.68 or 8.68% definitely improved the digestibility of dry matter and crude protein, while that of the ether extract and crude fibre decreased and there was only a slight positive influence on the digestibility of the N-free extract. Arrington et al. (1974) reported an improvement of the digestibility of the ether extract after addition of corn oil. Parigi-Bini et al. (1974) explain the contradictory results by an analytical difficulty, namely that the saponified fatty acids in the faeces cannot be detected by means of the commonly used analytical method. Consequently, a falsely good fat digestibility can be measured.

Lebas (1975*b*) in his recapitulative work connects the effect of fat supplementation with the energy concentration of the basal diet: the digestibility of feeds of low energy concentration will improve and that of high energy concentration will decrease after fat supplementation. On the other hand, on the basis of 19 digestibility trials testing unsupplemented raw materials, Fekete (1988) found a positive correlation among the digestibility of the ether extract (aDC, %), the ether extract content (x_1, %) and digestible energy concentration (x_2, MJ/kg) of the feed:

$$EE = 30.25 + 0.48x_1 + 3.28x_2;\ R^2 = 0.65$$

Schurg and Reid (1982) found the supplementation of a basal diet, very rich in energy and protein (14.83 MJ DE/kg, 19.7% crude protein), with 6% tallow ineffective both on the production trials and on the digestibility of protein and fibre. Partridge et al. (1986) supplemented a feed mixture of high energy and protein concentration with acidulated soybean oil and mixed animal fat. The digestibility of the crude protein did not change and that of the added lipids was found near to 100%. They applied a hydrochloric acid treatment before the chloroform–water–methanol solvent method of fat determination (Atkinson et al., 1972). Maertens et al. (1987) compared the fat digestibilities obtained by the different fat determinations (simple extraction with petroleum ether; previous HCl treatment). The simple extraction gave falsely high fat digestibility values. The other nutrients were not investigated.

A comparison of the results of the reviewed publications is very difficult, in some cases impossible, because the composition and nutritive value of the supplemented basal diets, the nature of the added fats and the analytical methods were different. The authors generally did not separate the effect of added, total and original lipid content of feeds and in the majority of cases the determination of digestibility did not comprise all the nutrients. The effect of ingested fat upon the lipid and cholesterol level of the blood plasma and on the fatty acid composition of blood lipids was not investigated.

The aim of the present experiment was to study the effect of animal fat and vegetable oil supplementation of feed mixtures of low and average energy concentration upon the digestibility of nutrients (crude protein, CP; ether extract, EE; total fat, TF; crude fibre, CF; and N-free extract, NFE) and the blood total lipid, phospholipid and cholesterol level, as well as the ratio of some fatty acids in the blood.

Materials and methods

Twenty, 4- to 5-month-old, female New Zealand White (NZW) rabbits weighing 3.29 $\pm$ 0.05 kg were involved in the experiment.

Period 1: the animals were divided into two groups (2×10) and the digestibility of nutrients in the feed mixture of medium (M) and low (L) energy concentration was measured.

Period 2: each group was divided into two subgroups and the digestibility of nutrients of the fat- and oil-supplemented feed of medium energy concentration (M+F and M+O, respectively) and that of low energy level (L+F and L+O, respectively) was determined.

Table I

Experimental design

Number of animals	Length of period	Applied feeds				Activity
		M: medium		L: low		Adjustment,
2×10	7+8 days	energy concentration 12.0 MJ DE 3.2% EE		8.5 MJ DE 3.3% EE		feeding, faeces collection, blood sampling at the end
4×5	10 days	M+F	M+O	L+F	L+O	Change of feeds, transition
4×5	8 days	M+F	M+O	L+F	L+O	Faeces collection, blood sampling at the end

M = basal diet of medium; L = basal diet of low energy level; MJ = megajoule; DE = digestible energy; EE = ether extract; F = 5% mixed animal fat (50% tallow + 50% lard) fat supplementation; O = 5% sunflower oil (destinated for human consumption) addition

Table II

Composition and calculated nutritive value of the experimental feeds

Composition, nutritive value, %	Experimental feeds					
	M	M+F	M+O	L	L+F	L+O
Alfalfa meal	30.0	30.0	30.0	87.0	86.0	88.0
Oat	32.5	22.5	27.5	—	—	—
Wheat	20.0	20.0	20.0	—	—	—
Sunflower meal	15.0	15.0	15.0	—	—	—
Favorit–50*	—	10.0	—	2.0	12.0	2.0
Sunflower oil	—	—	5.0	—	—	5.0
Wheat straw	—	—	—	10.0	1.0	4.0
Mineral–vitamin supplement	2.5	2.5	2.5	1.0	1.0	1.0
DE, MJ/kg	12.02	13.20	13.20	8.54	10.47	10.14
Crude protein, %	18.3	17.6	17.7	16.5	16.5	16.8
Ether extract, %	3.2	7.7	7.9	3.3	8.2	8.3
Crude fibre, %	14.9	13.7	14.3	25.4	21.2	22.0

* 50% maize meal, 25% beef tallow and 25% swine lard; M, M+F, M+O, L, L+F, L+O: see Table I

The length of the phases of the digestibility trials, the collection and storage of the faeces, the chemical analyses of the feed and faecal samples were carried out after Fekete and Gippert (1983). The experimental design is shown in Table I. Table II gives the composition and nutritive value of the feeds used.

The gross energy concentration of the feed and faecal samples was measured by an adiabatic bomb calorimeter (OQ-202), the total fat content was determined after Stoldt (1952), i.e. petroleum ether extraction of samples pretreated with 4 N HCl.

On days 15 and 33, i.e. at the end of the two digestibility trials, blood samples were collected from the v. marginalis and the total lipid (Zöllner and Kirsch, 1962), phospholipid (Baginski and Zak, 1960) and total cholesterol (Zlatkis et al., 1953) levels of the blood plasma were determined. The fatty acid composition of the plasma lipids was measured by gas chromatography, after extraction (Folch et al., 1957) and transformation into fatty acid methyl-ester (Husvéth et al., 1982).

The digestibility coefficients were calculated using the classical formula (Schürch, 1969). The statistical analyses were carried out as recommended by Pearce (1965).

Results

The changes of body weight and feed intake in the separate experimental periods are shown in Table III. The changes in body weight were very small and, except for the group consuming the oil-supplemented basal diet (M+O),

Table III

Data of feed intake and body weight changes (mean ± SEM)

Groups	Average daily feed intake, g DM/$W^{0.75}$	Difference, g*	Average daily weight gain, g	Difference, g*
M (to fat)	54 + 2.0		15.0 + 0.8	
M+F	50 + 2.0	−4.0	16.8 + 2.7	1.8
M (to oil)	53 + 2.0		10.9 + 3.0	
M+O	52 + 1.9	−1.0	9.8 + 2.0	−1.1
L (to fat)	61 + 2.4		9.5 + 1.8	
L+F	52 + 3.0	+1.0	9.8 + 2.0	+0.3
L (to oil)	58 + 2.5		7.6 + 1.0	
L+O	54 + 3.2	−4.0	7.0 + 1.6	−0.6

* Compared to the corresponding non-supplemented basal diet
DM = dry matter; W = body weight, kg
M, M+F, M+O, L, L+F, L+O: see Table I

were positive. Feed intake was reduced by the oil supplementation of both basal diets; in the case of fat supplementation, it was diminished only in rabbits given the feed mixture of medium energy concentration.

The digestibility coefficients of the nutrients of the applied feeds and the DE values are summarized in Table IV. The addition of animal fat to both basal diets improved the digestibility of the organic matter, that of sunflower oil only in the case of basal diet L. The digestibility of crude protein did not change definitely and significantly (0, −1, 0, −1 units). The digestibility of the ether extract from both basal diets significantly improved ($P < 0.001$), owing to the fat and oil supplementation (5, 9, 11, and 9 units).

The digestibility of crude fibre and N-free extract of the feed mixture of medium energy concentration did not change significantly when fat or oil was added to the diet. Fat or oil supplementation of feed L equally improved the digestibility of CF and NFE. The differences, except for one case, are significant. Totally, the digestible energy value was increased by 0.44 and 1.99 MJ/kg owing to animal fat and by 0.89 and 2.22 MJ/kg owing to sunflower oil addition to feed M and L, respectively.

Table V shows the data of fat contents, digestibility coefficients and DE values, obtained by the classical and total fat determination. The total fat level is higher in both the feed and the faecal samples than that of the ether extract. The digestibility coefficients, calculated from the total fat content, are lower. Nevertheless, the DE values differ significantly only between the two basal diets.

The data of the blood analyses are summarized in Table VI. The total lipid, phospholipid and cholesterol levels of the blood of rabbits consuming one

Table IV

Apparent digestibility coefficients, % (aDC) of nutrients and the measured digestible energy (DE) contents of diets (mean ± SEM)

Parameters		M-I	M+F	M-II	M+O	L-I	L+F	L-II	L+O
Effectives		5	5	5	5	5	5	5	5
aDC of DM		72	74	72	72	56	61**	56	64***
	±	0.7	0.2	0.6	0.7	0.7	0.8	0.4	1.3
aDC of ash		44	49	45	49	60	65**	63	65
	±	3.0	1.6	1.8	0.9	1.3	1.1	1.0	1.6
aDC of OM		74	75	74	74	55	60**	56	64***
	±	0.7	0.2	0.6	0.7	0.7	1.4	0.7	1.3
aDC of CP		83	83	83	82	71	70	71	71
	±	0.9	0.5	0.6	0.5	0.8	1.8	0.6	1.7
aDC of EE		84	89***	82	91***	72	83***	70	79***
	±	0.3	0.8	0.5	0.4	1.1	0.4	1.0	1.2
aDC of TF		71	79**	66	84***	64	59*	64	76**
	±	0.8	0.6	0.7	0.6	1.0	0.7	0.8	1.0
aDC of CF		28	28	33	27	22	26	17	45***
	±	2.1	0.5	1.2	2.6	1.3	2.1	1.9	1.5
aDC of NFE		83	81	80	79	63	70**	64	69**
	±	0.4	0.3	0.6	0.5	0.9	1.0	0.9	1.5
DE-I, MJ/kg		12.77	13.21	12.56	13.45	9.26	11.25	9.11	11.33
DE-II, MJ/kg		12.36	13.09	12.03	13.34	8.99	10.78	9.37	11.13

M-I and L-I = basal diets to animal fat, M-II and L-II = basal diet to sunflower oil supplementation; DM = dry matter; OM = organic matter; CP = crude protein; EE = ether extract; TF = total fat; CF = crude fibre; NFE = N-free extract; DE-I and DE-II = energy values, calculated by the ether extract (I) or total fat (II) content of feed and faeces *' **' *** $P < 0.05$, 0.01 and 0.001, respectively, to the corresponding basal diet

Table V

Comparison of the parameters based on ether extract (I) and on total fat (II) determination (mean ± SEM)

Parameters	Total pool	Basal diets	Supplemented diets
Feed EE level, %	5.76 ± 0.81	3.56 ± 0.59	6.85 ± 0.61
Feed TF level, %	7.33 ± 1.18	4.52 ± 0.96	8.73 ± 1.18
Faeces EE level, %	2.98 ± 0.16	2.52 ± 0.14	3.44 ± 0.25
Faeces TF level, %	5.97 ± 0.33	4.63 ± 0.10	7.32 ± 0.47
aDC of EE, %	80.50 ± 1.21	76.20 ± 1.57	84.60 ± 1.20
aDC of TF, %	69.90 ± 1.38	65.90 ± 0.79	73.80 ± 2.30
DE-I, MJ/kg	11.44 ± 0.61	9.55 ± 2.00	12.13 ± 0.57
DE-II, MJ/kg	11.39 ± 0.57	10.69 ± 0.88	12.09 ± 0.66

of the two basal diets did not differ significantly. On the other hand, the proportion of the fatty acids was not the same, namely the blood of rabbits fed the basal diet of medium energy concentration was more abundant in myristic, palmitic and oleic acids and poorer in stearic, linoleic, linolenic and arachidonic acids.

Sunflower oil supplementation of feed M, first of all due to palmitic acid and oleic acid, increased the proportion of stearic and linoleic acids. Besides, the addition of animal fat increased the quantity of linolenic and arachidonic acids.

The supplementation of feed L gave different results. Animal fat addition increased mainly the ratio of oleic and linoleic acid, in the first place on the account of linoleic acid. Oil supplementation raised only the proportion of linoleic acid significantly; the increase of myristic, margarinic and oleic acid ratios was negligible. The concentration of the other fatty acids decreased.

Discussion

In the present work a basal diet of medium and one of low energy concentration (12.02 and 8.54 MJ DE/kg, respectively), both of medium ether extract level (3.2 and 3.3%), were supplemented with 5% mixed animal fat or sunflower oil.

According to previous data (Lebas, 1975*a*), the voluntary feed intake decreased in the majority of cases owing to such supplementations. However, the consumption of feed poor in energy slightly increased if supplemented with fat. The difference can be explained by the better quality of the pellet (improved palatability, reduced powder formation, etc.). Since the rabbits were practically adults (body weight gain: 1–2 g/animal/day), far-reaching conclusions cannot be drawn.

Table VI

Some lipid parameters of the blood plasma and its fatty acid composition in % of the total fatty acids (mean ± SEM)

Parameters		M-I	M+F	M-II	M+O	L-I	L+F	L-II	L+O
Effectives		4	4	4	4	4	4	4	4
Total lipids, g/l		3.56	4.19	2.97	2.96	3.40	4.50	3.43	3.35
	±	0.48	0.38	0.17	0.35	0.39	0.72	0.48	0.46
Phospholipids, mg/l		836	679	769	777	860	888	888	972
	±	178	101	107	151	358	281	279	155
Total cholesterol, mmol/l		2.35	2.41	1.86	1.90	2.28	3.34	2.15	2.17
	±	0.14	0.14	0.13	0.07	0.30	0.32	0.33	0.10
C12:0, %		0.2	0.1	0.2	0.2	0.2	0.2	0.3	0.1
	±	0.1	0.03	0.1	0.1	0.2	0.04	0.1	0.1
C14:0, %		2.4	1.6	2.5	1.9	2.0	1.8	1.8	2.0
	±	0.5	0.1	0.5	0.4	0.5	0.3	0.5	1.4
C15:0, %		0.7	0.5	0.7	0.6	0.7	0.7	1.0	0.7
	±	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
C16:0, %		27.9	25.7	26.7	23.7	22.9	24.6	25.9	21.8
	±	2.0	1.8	2.1	2.6	2.4	1.7	3.3	1.0
C16:1, %		2.4	2.4	3.2	1.9	2.1	2.4	1.8	1.3
	±	1.5	0.4	0.3	0.6	0.8	0.4	0.7	0.3
C17:0, %		0.7	0.7	0.8	0.7	1.1	1.0	1.2	1.0
	±	0.2	0.1	0.03	0.1	0.1	0.1	0.2	0.2
C18:0, %		9.3	11.4	9.5	11.3	12.6	11.3	12.5	12.9
	±	1.8	0.2	0.7	1.0	0.9	1.4	2.3	1.6
C18:1, %		30.5	27.9	29.8	23.5	19.8	24.9	17.5	18.1
	±	3.3	1.6	2.9	2.8	2.6	1.8	2.4	1.6
C18:2, %		22.2	24.8	22.8	32.8	30.4	21.5	28.9	35.4
	±	3.5	2.7	2.8	5.3	2.8	1.9	2.8	1.6
C18-3, %		1.9	2.1	1.7	1.6	5.4	9.3	6.8	4.8
	±	0.4	0.3	0.3	0.2	1.5	1.7	1.5	1.1
C20:4, %		1.9	2.7	2.0	1.9	2.7	2.3	2.4	2.1
	±	0.4	0.7	0.2	0.5	0.7	0.6	0.8	0.7

C12:0 = lauric; C14:0 = myristic; C15:0 = pentadecanoic; C16:0 = palmitic; C16:1 = palmitoleic; C17:0 = margarenic; C18:0 = stearic; C18:1 = oleic; C18:2 = linoleic; C18:3 = linolenic and C20:4 = arachidonic acid.

For the other abbreviations see the previous tables

Fat supplementation significantly ($P < 0.001$) increased the digestibility coefficient of the ether extract in the case of both basal diets; the digestibility of crude fibre and N-free extract improved only in the group given basal diet L. The changes seen after oil supplementation showed the same tendency, but numerically were greater. The improvement found in the digestibility of the ether extract can be imagined only if the digestibilities of the added fat and oil were very high, close to 100%. Similar results were reported by Arrington et al. (1974), Parigi-Bini et al. (1974), Partridge et al. (1986) and Maertens et al. (1987). In our opinion, the low fat digestibilities, measured by Teleki and Darwish (1969) and Lebas (1975*a*), can presumably be attributed to some technical reasons (degree of homogeneity, carrier of added fat and oil, etc.).

The digestibility coefficients for the total fat are generally lower than those for the ether extract (Table IV).

In our case practically neither animal fat nor sunflower oil supplementation influenced the digestibility of protein. This finding is in good agreement with that of Schurg and Reid (1982) and Partridge et al. (1986). The improvement of protein digestibility reported by Teleki and Darwish (1969), in our opinion, might be attributed to the low energy concentration of the basal diet used in their experiment.

The tendency of our data confirms Lebas's statement (1975*b*) that the effect of fat supplementation upon the digestibility of nutrients depends on a large scale on the energy concentrations of the basal diet without fat supplementation. In our case not only the supplementation of the diet of low energy concentration, but also that of the diet of medium energy level exerted a positive effect on the digestibility of nutrients. The latter improvement, however, was less pronounced. Namely, the digestible energy concentration of feed L rose by 21.5 and 24.4% (1.99 and 2.22 MJ DE/kg) owing to the added fat and oil, respectively. The corresponding numbers for the diet of medium energy concentration are 3.4 and 7.1% (0.44 and 0.89 MJ DE/kg). The reason for the differences is the dissimilar digestion of the crude fibre and N-free extract.

If one calculates the DE values using the digestible total fat content (Table V), the results will be significantly higher (by 1.14 MJ DE/kg, i.e. 11.9%) only for the basal diets. On the basis of Table V and the previous statement, i.e. that the added fat and oil are very well digested, total fat determination is reasonable to get to know the real fat digestibility coefficients of feed mixtures and to permit an evaluation of the digestible energy concentration of feed ingredients.

Although the initial total lipid level of the blood was practically the same (3.42 *vs.* 3.27 g/l) in the two basal groups, the serum composition reflected well the nature of the consumed feed. The blood plasma of rabbits eating the feed of low energy concentration contained essentially less myristic, palmitic and oleic but more stearic, linoleic, linolenic and arachidonic acids. Animal fat

addition to the diet increased the total lipid concentration (by 18 and 32%) in both groups without supplementation; the supplementation of sunflower oil had no similar effect. Neither animal fat nor vegetable oil addition influenced the cholesterol level of the blood plasma, which was, however, in the physiological range (Magdus et al., 1988). After the intake of the applied quantity of fat and oil, presumably the regulatory mechanisms described by Massaro and Zilversmit (1977) and Goldstein et al. (1983) prevent the rise of the blood cholesterol level. The plasma cholesterol concentration of healthy, non Watanabe heritable hyperlipaemic rabbits will increase only owing to feeding a larger amount of fat, especially if it is given together with cholesterol (Whatley et al., 1981).

Both supplementations modified the composition of plasma lipids. The effect of the added fat was different from that of the added oil, and the changes depended upon the nature of the basal diet, too. It is characteristic of each treatment that the ratio of biologically valuable polyunsaturated fatty acids increased. On the basis of these results, the supplementation of rabbit feeds with fat or oil can be justified if the hygienic respects (rancidity) and nutrient balance are considered.

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TREATMENT OF ANOESTROUS MARES WITH A SYNTHETIC PROGESTAGEN, ALLYLOESTRENOL

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Anoestrous mares were treated with prostaglandin (n = 43) and those that did not respond to prostaglandin (n = 29) with a synthetic progestagen, allyloestrenol, at a dose of 0.05 mg/kg body mass for 12 days. After the cessation of the long-term per os gestagen blockade the animals were checked for heat and, if a preovulatory follicle could be palpated, 2000 IU hCG was administered to induce ovulation. In some animals the plasma 17β-oestradiol (E_2) and progesterone (P_4) levels were also followed up throughout the gestagen treatment and for 10–14 days thereafter. As the favourable oestrus rate (86.2%) and pregnancy rate (65.5%) indicate, the 12-day allyloestrenol treatment seems to be a reliable tool for induction of cycling in mares anoestrous early in the season. These results, supported by the E_2 and P_4 profiles, suggest that an important reason for anoestrus in mares is the absence of a functional corpus luteum that can be substituted for by exogenous gestagen administration. The timing of ovulation, however, should still be improved.

Keywords: Mare, anoestrus, per os progestagen treatment.

The current therapy of equine anoestrus remains unsatisfactory. Although many mares which are anoestrous in late winter and early spring do start cycling later in the season, the owners wish to have their animals mated or inseminated as early as possible. The primary class of mares that requires attention during anoestrus and the transitional period is that of barren or open mares. This seems to be especially valid for those mares that do not show any signs of heat early in the breeding season.

Anoestrus can be of various origin, such as influence of the weather, failures in central nervous stimuli to the ovaries, etc. In the affected animals small and torpid ovaries with or without small follicles that hardly develop can be palpated. Naturally occurring prolongation of luteal activity in the non-pregnant mare is one of the most important causes of infertility in the horse. The condition may persist for 30–90 days and may include endometritis as a sequel.

In some cases large follicles may be palpated that show no development. In the mare the transitional period is known to represent a condition which often involves follicular growth that does not end in ovulation. It is not uncom-

mon for mares to bring up 2 or 3 sets of follicles before ovulation occurs, probably due to the inability of the hypothalamus and anterior pituitary to respond to the positive feed-back effect of oestrogen to initiate the preovulatory LH surge. Once, however, a mare has ovulated, ovulatory failure almost never occurs in that season (Stabenfeldt and Hughes, 1987).

In their recent publication Fitzgerald et al. (1987) concluded that treatment of seasonally anoestrous mares and problem acyclic mares during the breeding season with an LHRH agonist is a reliable method of inducing ovulation and, more importantly, in the absence of conception a high proportion of mares subsequently exhibit oestrous cycles. The regimen which involves two intravenous treatments daily for 10–14 days seems to be less practical.

Apart from the direct stimulation of follicular growth and ovulation with GnRH and gonadotropins that are of limited value in inducing heat and reaching pregnancy, the prostaglandins are now regarded as the treatment of choice for all forms of prolonged dioestrus (Gordon, 1983). By their luteolytic effect, the prostaglandins induce regression of the corpus luteum, blocking the cycle in the luteal phase.

According to Allen and Alexeev (1980), it is probably the absence of a functional corpus luteum in the ovaries of the anoestrous mare, the regression of which would normally trigger the release of sufficient LH to bring about follicular maturation and ovulation, which prolongs the non-breeding period.

Progesterone and progestagens are known to exert a powerful negative feed-back action on the release of LH. A sharp increase in pituitary LH secretion is regarded as an essential prerequisite of the first ovulation of the new breeding season (Freedman et al., 1979).

Results with a synthetic progestagen, allyl trenbolon, over a period of 10–15 days showed that this was a practical means of hastening the onset of the breeding season in mares if they are in a transitional phase between anoestrus and the breeding season. It cannot be employed in mares in deep anoestrus (Palmer, 1979; Heeseman et al., 1980) unless they are shifted from deep anoestrus into the transitional phase by artificial or natural lighting (Scheffrahn et al., 1980).

Loy et al. (1981) suggested a combined regimen which involves daily doses of 150 mg progesterone and 10 mg oestradiol for 10 days with a luteolytic prostaglandin given at the end of this treatment. This may decrease the variability in follicular development found at the end of treatment when progesterone alone is employed.

A long-lasting progestagen treatment inhibits the release of gonadotrop hormones, especially that of LH. After sudden cessation of gestagen administration an intensive gonadotropin surge, called the rebound effect, will stimulate the ovarian functions. This promises higher efficacy compared to a direct pharmacological effect.

In a recent publication, Waelchli (1987) summarizes the different hormonal treatments including those with gestagens, used for regulating equine reproduction.

The aim of the present trial was to study the possible use of a synthetic progestagen, allyloestrenol, for treating anoestrous mares that do not respond to prostaglandins. This idea was supported by a preliminary pilot experiment in which anoestrous mares started cycling after oral administration of this drug for 12 days (Solti et al., 1984).

Materials and methods

Mares from a horse stud and some single mares from state farms or private farms were involved in the study, with special attention to those that remained barren early in the season. After gynaecological examination directed to the reproductive stage and soundness of the genital organs of these mares, they were first treated with a luteolytic dose of one of the following prostaglandin preparations: 5 mg dinoprost (Enzaprost, Chinoin, Hungary), 250 μg cloprostenol (Oestrophan, Spofa, Czechoslovakia or Cloprostenol, Jenapharm, GDR) or 250 μg fluprostenol (Alestrum, Spofa, Czechoslovakia). In the case of endometritis intrauterine treatment was applied prior to, or together with, the administration of prostaglandin. Animals showing heat following the prostaglandin injection were teased daily and mated upon standing oestrus.

To those mares that did not respond to prostaglandin within 10 days, allyloestrenol (Turinal 5 mg tablets, G. Richter, Hungary) was administered for 12 days at a dose of 5 mg per 100 kg body mass. Thereafter, all treated animals were checked twice daily for heat signs and their ovaries controlled for the presence of follicles by rectal palpation. For induction of ovulation 2000 IU hCG (Gonabion, Jenapharm, GDR) was applied intramuscularly if follicle size exceded 30 mm in diameter. The mares were then mated and checked for returning to heat until pregnancy was confirmed.

From the jugular vein of some of the experimental animals blood was collected into heparinized tubes and plasma samples were harvested for progesterone and 17β-oestradiol analysis. These plasma samples were stored at -20 °C until assayed.

Plasma 17β-oestradiol was determined in the Central Isotope Laboratory of the KMU Leipzig by radioimmunoassay after diethyl ether extraction. The antibody was raised against 17β-oestradiol-6-carboxymethyloxime in rabbits, the tracer used was ^{3}H-labelled 17β-oestradiol, and the bound and the free fractions were separated with dextran-coated charcoal.

Plasma progesterone was assayed by direct microplate ELISA based on the method published elsewhere (Solti et al., 1986). Briefly, the Dynatech

B plates were coated overnight by 150 μl/well diluted antibody raised in rabbits against 11α-OH-P-HS-BSA (11α-hydroxyprogesterone hemisuccinate–bovine serum albumin) immunogen using a coating buffer of pH 9.6. After washing, 20 μl standard or sample and 150 μl 11α-OH-P-HS-horseradish peroxidase conjugate were added to the wells. After incubating the plate for 1 h at room temperature, the wells were emptied and washed 3 times with 0.05%. Tween 20 in PBS, then 150 μl H_2O_2-orthophenylenediamine substrate solution was added. After 15 min in the dark at room temperature the reaction was stopped with 50 μl 6N sulphuric acid and the absorbance measured at 492 nm.

Results

Figure 1 and Fig. 2 show the effect of allyloestrenol treatment as reflected by the plasma progesterone and oestradiol profiles. In the case of "Silke" and "Nelli" the progesterone levels remained low throughout the treatment, while the oestradiol profiles indicate some anovulatory follicular development during the gestagen blockade. After the withdrawal of the allyloestrenol tablets new preovulatory oestradiol pulses are seen that are supported by hCG administration.

The mating of "Silke" (Fig. 1) on day 17 (day 0 = start of gestagen administration), i.e. 5 days after withdrawal of the tablets and 24 h following the hCG administration, seems to have been successful as verified later by rectal palpation.

Some animals, such as "Nelli" (Fig. 2), that failed to exhibit definite oestrous signs within 5 days after treatment, may come into heat 10–15 days

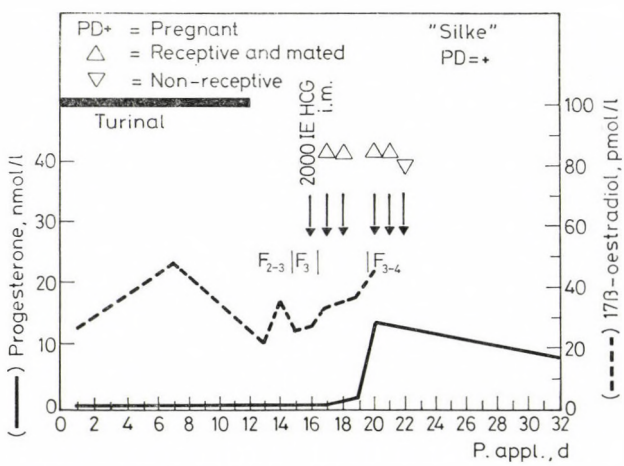

Fig. 1. E_2 and P_4 profiles of "Silke" who came in heat 5 days after treatment and conceived

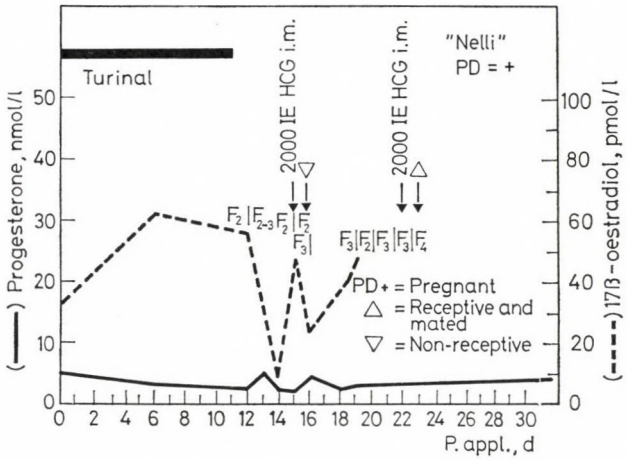

Fig. 2. E_2 and P_4 profiles of "Nelli" who showed oestrous signs on day 7 after treatment

later. "Nelli" showed standing heat neither immediately after the gestagen treatment nor following the hCG injection. The follicle that could be palpated on the left ovary remained anovulatory and only on day 19, parallel to the growth of a new follicle on the right side, did this mare show standing oestrus with ovulation that resulted in pregnancy.

In the case of "Domäne" (Fig. 3) there was also no standing heat after the treatment, though, at least according to the plasma progesterone profile, a relatively less developed follicle may have ovulated without external signs of oestrus. The subsequent luteal phase was somewhat shortened and led to

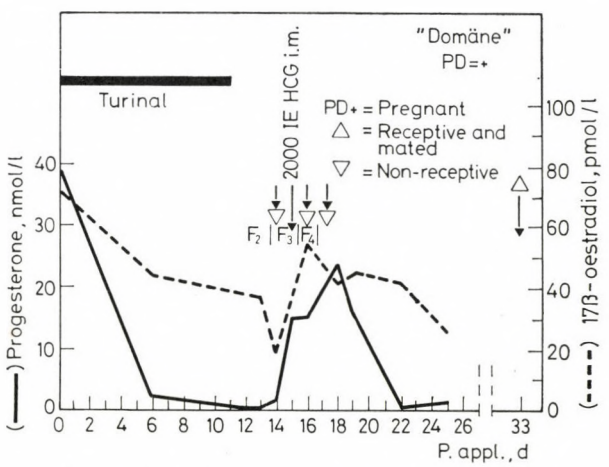

Fig. 3. "Domäne" had functional corpus luteum at the start of gestagen treatment that regressed thereafter

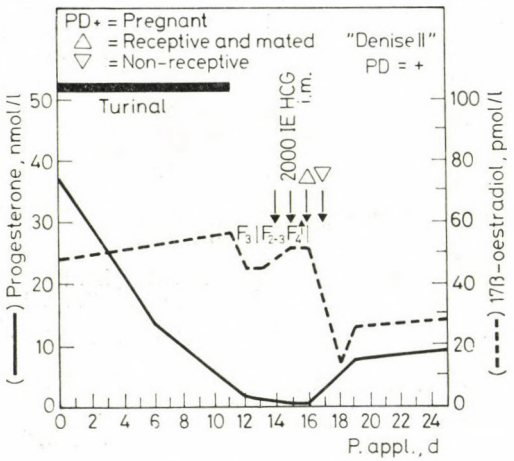

Fig. 4. Case history of "Denise II" during and after the allyloestrenol treatment

a spontaneous heat on day 33. This was used for mating which resulted in pregnancy.

Neither "Domäne" (Fig. 3) nor "Denise II" (Fig. 4) showed any oestrous signs after prostaglandin administration. However, they had a functioning corpus luteum at the beginning of allyloestrenol treatment which regressed during the gestagen blockade, as verified also by the plasma progesterone profile. "Denise II" showed, however, a regular oestrus after withdrawal of the allyloestrenol tablets and conceived. This indicates that progestagen treatment should not inevitably be preceded by luteolytic prostaglandin injection.

Table I shows that the oestrus rate and conception rate of mares that were considered problem animals are satisfactory. Two-thirds of the allyl-

Table I

Oestrus and pregnancy rates of mares treated with prostaglandin or after gestagen blockade

Fertility parameters	Treatment	
	Luteolysis with prostaglandin n = 43 (100%)	Gestagen blockade with Turinal n = 29 (100%)
Oestrus rate, n (%)	33 (76.7)[1]	25 (86.2)[2]
Pregnancy rate after 1st mating, n (%)	15 (34.9)	10 (34.5)
Overall pregnancy rate, n (%)	18 (41.9)	19 (65.5)

[1] 93% in mares with corpus luteum verified by plasma progesterone determination;
[2] 69% within 7 days

oestrenol-treated mares became pregnant following the gestagen treatment, 34.5% of them after the first mating, which in our experiment was superior to the conception rate of the prostaglandin-treated animals.

Discussion

The low plasma progesterone level of "Silke" and "Nelli" at the beginning of the treatment is in good agreement with the hypothesis of Allen et al. (1980) who believe the absence of a functioning corpus luteum to be a cause of anoestrus in the mare. While progesterone remained on the baseline level throughout the gestagen administration, the oestradiol profiles indicate some follicular activity already during the treatment.

In spite of the follicular activity during gestagen administration, some animals, e.g. "Nelli" did not show any definite signs of heat within 5 days after the cessation of the treatment. They came, however, into standing oestrus 10–15 days later, which seems to support the findings of Stabenfeldt and Hughes (1987) concerning the development of 2 or 3 sets of follicles before an ovulation occurs after the transitional period. In anoestrous mares, the exogenous gestagen may substitute the beneficial effect of a non-existing corpus luteum which would induce the LH surge needed for follicular maturation after a non-breeding period.

We found that the measuring of 17β-oestradiol and progesterone levels in the plasma is a useful tool for monitoring ovarian activity even if it is not accompanied by behavioural oestrous symptoms. This experience is in good accordance with recent conclusions of King et al. (1988) who could predict impending anoestrus in the mare from the declining pattern of dioestrous progesterone concentrations. Pool-Anderson et al. (1988) also used radioimmunoassay of oestradiol and progesterone for monitoring the endocrine events after the administration of an exogenous hormone regimen.

The corpora lutea that "Domäne" and "Denise II" had at the beginning of allyloestrenol treatment regressed during the gestagen blockade. In view of this, luteolytic pretreatment is not absolutely necessary prior to gestagen administration. On the other hand, from an economic point of view, pretreatment for selecting the anoestrous mares with a persisting corpus luteum that respond well to prostaglandin is preferred.

According to the favourable overall conception rate of problem mares shown in Table I the gestagen blockade applied in our trial seems to be more effective than oestrus induction with luteolytic agents. Most of our allyloestrenol-treated mares had been unsuccesfully pretreated with some prostaglandin preparation, thereby the oestrus and ovulation that followed allyloestrenol administration should be regarded as a consequence of the substitution effect of the gestagen.

The great variability of the hormonal responses is characteristic of the equine species. Although our experience concerning the 12-day allyloestrenol treatment of anoestrous problem mares is very favourable, the timing of ovulation should be improved. In this study 2000 IU hCG was used which regimen is known to induce ovulation between 24 and 48 h (Sullivan et al., 1973), but repeated administration may lead to production of antibodies against

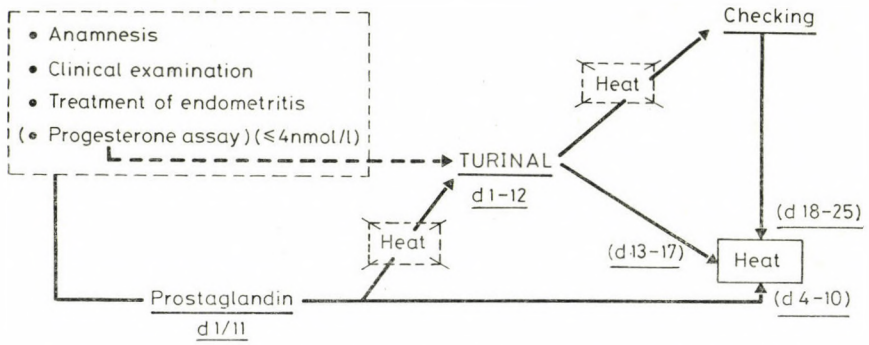

Fig. 5. Flow chart of the therapy recommended for mares anoestrous early in the breeding season

hCG. Others, therefore, use new approaches for induction of ovulation, such as crude horse gonadotropin (Duchamp et al., 1987) or a synthetic prostaglandin, fenprostalene, during oestrus (Savage and Liptrap, 1987). These efforts show that none of the above-mentioned methods is reliable enough in timing the ovulation.

In view of our recent findings, the following therapy scheme is recommended for the treatment of anoestrus mares (Fig. 5). With knowledge of the case history and the result of a thorough clinical examination, supported eventually by blood progesterone assay, a luteolytic prostaglandin treatment should be performed first. Only animals that fail to respond with oestrus to the prostaglandin should be treated with gestagen. After withdrawal of the gestagen an oestrus is expected between day 13 and 17, therefore the mare should be checked for heat daily. Then 2000–3000 IU hCG is given to those mares that have an ovulatory follicle in one of the ovaries.

The possibility of shortening the rather long gestagen treatment regimen to only 6 days as well as of improving the induction of ovulation should be cleared up in later experiments.

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A SYNTHETIC, SELECTIVE CULTURE MEDIUM FOR *PSEUDOMONAS AERUGINOSA*

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Selective and differentiating media consisting of simple chemical components have been developed, both in solid and in liquid form, for culturing *Pseudomonas aeruginosa* from foodstuffs.

These media work on the basic principle that Gram-negative bacteria can utilize ammonia as inorganic nitrogen source. This principle has already been utilized for developing a culture medium for coliform bacteria (Szita and Biró, 1986; Szita et al., 1988).

P. aeruginosa can produce the ammonia needed for its growth by decomposing acetamide.

The liquid synthetic medium was compared with the nitrofurantoin broth and the solid one with cetrimide agar by parallel inoculation of 60 raw milk samples and 20 *P. aeruginosa* pure cultures.

The main advantages of the synthetic media are their high selectivity, high sensitivity and rapidity. Owing to their advantageous properties, the new media can be recommended for replacing cetrimide agar and nitrofurantoin broth.

Keywords: Inhibitory substance, selective medium, synthetic medium, *Pseudomonas aeruginosa.*

Pseudomonas aeruginosa is a ubiquitous bacterium species. It occurs in the faeces of animals, in feed, drinking water and also in foodstuffs. It is involved in the aetiology of mastitis. As the microbiological examination of foodstuffs often reveals the presence of *P. aeruginosa*, the performance of the applied culturing method is of great importance.

Standardized media (Hungarian Standard 3640/7-80) used for culturing *P. aeruginosa* usually contain potent inhibitory substances which not only suppress the competing bacterial flora but exert an adverse effect on the bacteria to be demonstrated, too. Therefore, a synthetic culture medium free from inhibitory substances was made up which works on the principle that *P. aeruginosa* grows well in a medium containing ammonia as nitrogen source and glucose as carbon source (Microorganisms in Foods, Vol. 1, 1978; Bergey, 1974). As most bacteria are able to utilize glucose, it should be replaced with acetate which only bacteria with oxidative properties can use up. To achieve as good selective effect as possible, we looked for a substance which contained these compounds in chemically bound form. Acetamide was found to be the simplest such compound which contains ammonia and acetate in an acid–amide bond. As very few bacteria can split acetamide to its components, for

most bacteria acetamide is not suitable as growth medium. *P. aeruginosa*, on the other hand, releases from acetamide ammonia and acetic acid, and thus produces the nutrients needed for its own growth. The working principle of the new synthetic medium is our recognition that acetamide in itself is sufficient to support the growth of *P. aeruginosa*. As sulphur compounds are also necessary for the production of bacterial proteins, the medium should be supplemented with sulphate ions. Bacteria need many times more carbon atoms than nitrogen for building up their body: therefore, part of the produced ammonia accumulates and the pH shifts in alkaline direction. This pH shift can be prevented by adding phosphate buffers. Potassium ions facilitate pigment production (Horváth, 1980; Kiss, 1974).

Materials and methods

For one of the most important biochemical identification procedures, the complete nitrate reduction test potassium nitrate was added to the synthetic liquid medium suitable for culturing *P. aeruginosa* (further on referred to as "Z broth"). Thus, primary isolation of the bacterium from the sample and nitrate reduction may occur at the same time, which saves time and medium as compared with the nitrofurantoin broth specified in the standard (Hungarian Standard 3640/7-80). At the same time, nitrate reduction in the synthetic medium does not take place without the mediators necessary for oxidation-reduction (redox) reactions. In such cases, thiosulphate ions or elemental sulphur are suitable for electron transfer (Horváth, 1980). Therefore, the medium had to be supplemented also with one of these substances. The nitrogen produced during nitrate reduction was collected in Durham's fermentation tubes. When using the solid medium, gas production cannot be detected; therefore, no nitrate was added to that medium.

Z broth was compared with the nitrofurantoin broth by the MPN method (Hoskins, 1934), using dilutions of *P. aeruginosa* strain 170001 (10 samples).

The synthetic solid medium developed for culturing *P. aeruginosa* (further on termed "Z agar") was compared with cetrimide agar. Common agar was used as control. Dilutions of the above test bacterium strain were used (10 samples).

Sixty raw milk samples were taken from milk coolers at dairy farms and inoculated onto Z agar and cetrimide agar to permit comparison of the media using, in addition to pure bacterial cultures, also test materials of mixed microflora. The colonies were identified by the oxidase and nitrate reduction test and on the basis of oxidative and fermentative glucose splitting, urea splitting, and pigment production.

Parallel inoculations were used in all test series to determine the possible methodological errors of the given method and to permit a mathematical-statistical evaluation (Weber, 1971). In the first step of the biometric calculations, the means and standard deviations of logarithmic values of the parallel inoculations were determined. Subsequently the means and standard deviations were added up for all test samples and for all media used in the experiment. The homogeneity of the standard deviations was checked by Fischer's F test. The significance calculations were done by the two-sample t test and, at a given degree of freedom, the results were compared with the value given in Student's t table at a probability limit of 95% (Hajtman, 1968; Hajtman, 1977).

The biometric calculations were performed with an ENTERPRISE 128 type computer.

To check the selectivity of the media, the nitrofurantoin broth and the Z broth were inoculated with pure cultures of test bacteria. The test bacteria included *Pseudomonas* spp., bacteria belonging to different genera of the Enterobacteriaceae family, and different Gram-positive species (Table II).

Results

The mean logarithmic value obtained for nitrofurantoin broth and Z broth inoculated with pure bacterial cultures was 1.58 and 1.57, respectively (Fig. 1). The methodological source of error, obtained by adding up the standard

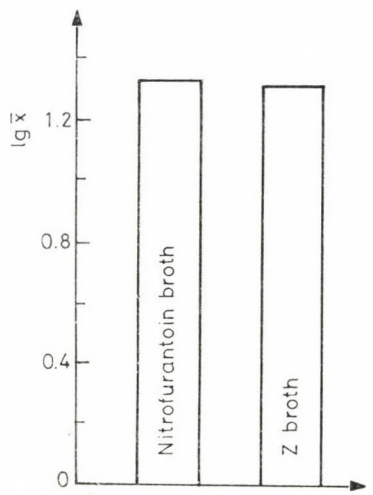

Fig. 1. Microbe counts obtained in nitrofurantoin and Z broth inoculated with dilutions of the broth culture of *Pseudomonas aeruginosa* strain 170 001

Table I

Comparison of *Pseudomonas aeruginosa* counts cultured from raw milk samples on cetrimide agar and Z agar

Sample no.	Cetrimide agar		Z agar	
	a	b	a	b
1	1.78	<1.00	1.00	<1.00
2	1.78	2.28	1.95	1.84
3	<1.00	2.48	<1.00	<1.00
4	1.30	<1.00	1.30	1.48
5	<1.00	<1.00	2.00	<1.00
6	<1.00	<1.00	1.60	1.30
7	<1.00	<1.00	1.30	1.69
8	<1.00	<1.00	2.32	2.20
9	<1.00	<1.00	2.20	2.15
10	<1.00	<1.00	2.60	2.30
11	<1.00	<1.00	3.17	2.69
12	<1.00	<1.00	2.43	1.84
13	<1.00	<1.00	2.62	2.34
14	<1.00	<1.00	2.84	2.78
15	<1.00	<1.00	2.90	<1.00
16	<1.00	<1.00	3.06	1.00
17	<1.00	<1.00	1.47	2.50
18	1.00	1.00	2.48	2.84
19	1.30	1.30	3.52	3.43
20	1.84	1.95	3.91	3.91
21	1.00	1.78	4.13	4.02
22	1.60	<1.00	3.52	3.72
23	2.32	1.00	3.23	3.46
24	<1.00	2.11	2.20	1.90
25	1.95	<1.00	1.95	2.28
26	<1.00	<1.00	2.30	2.30
27	<1.00	<1.00	<1.00	2.48
28	2.04	1.60	2.58	2.47
29	2.00	2.23	2.62	2.61
30	<1.00	<1.00	2.30	2.78
31	<1.00	<1.00	2.30	3.04
32	1.90	2.34	3.03	2.94
33	2.46	2.75	2.75	2.79
34	<1.00	<1.00	2.48	2.84
35	<1.00	<1.00	2.48	2.84
36	<1.00	1.00	2.25	1.95
37	<1.00	1.60	1.90	2.17
38	<1.00	<1.00	2.00	2.30
39	1.00	<1.00	<1.00	<1.00
40	<1.00	<1.00	1.00	1.30
41	<1.00	<1.00	1.00	<1.00
42	<1.00	<1.00	<1.00	2.00
43	<1.00	<1.00	1.00	1.00
44	<1.00	<1.00	1.60	1.69
45	<1.00	<1.00	<1.00	2.00

Table I continued

Sample no.	Cetrimide agar		Z agar	
	a	b	a	b
46	<1.00	1.00	1.60	1.84
47	<1.00	<1.00	1.48	1.48
48	<1.00	<1.00	1.00	1.00
49	<1.00	<1.00	1.69	1.60
50	<1.00	<1.00	<1.00	1.69
51	<1.00	<1.00	1.00	<1.00
52	<1.00	<1.30	1.30	1.48
53	1.00	<1.00	1.00	<1.00
54	<1.00	<1.00	1.00	1.00
55	1.00	<1.00	1.00	1.30
56	<1.00	1.00	1.00	1.30
57	<1.00	<1.00	1.00	<1.00
58	<1.00	<1.00	1.00	<1.00
59	<1.00	<1.00	1.30	1.00
60	<1.00	<1.00	1.00	<1.00

deviations of the parallel inoculations, was 0.220 and 0.390, respectively. The F value calculated from the former was 3.14, lower than the value given in the table (3.18); thus, the standard deviations can be considered homogene-

Table II

Comparison of the nitrofurantoin broth and the synthetic (Z) broth after inoculation with test bacteria

Test bacterium	Nitrofurantoin broth	Z broth
Pseudomonas aeruginosa	+	+
Pseudomonas acidovorans	—	—
Pseudomonas cepacia	—	—
Pseudomonas mendocina	+	—
Pseudomonas putrefaciens	—	—
Pseudomonas putida	—	—
Pseudomonas testosteroni	—	—
Pseudomonas maltophilia	—	—
Pseudomonas diminuta	—	—
Bacillus cereus	—	—
Staphylococcus aureus	—	—
Micrococcus luteus	—	—
Escherichia coli	—	—
Enterobacterium aerogenes	—	—
Klebsiella pneumoniae	—	—
Citrobacter freundii	—	—
Salmonella enteritidis	—	—
Proteus vulgaris	—	—
Yersinia enterocolitica	—	—

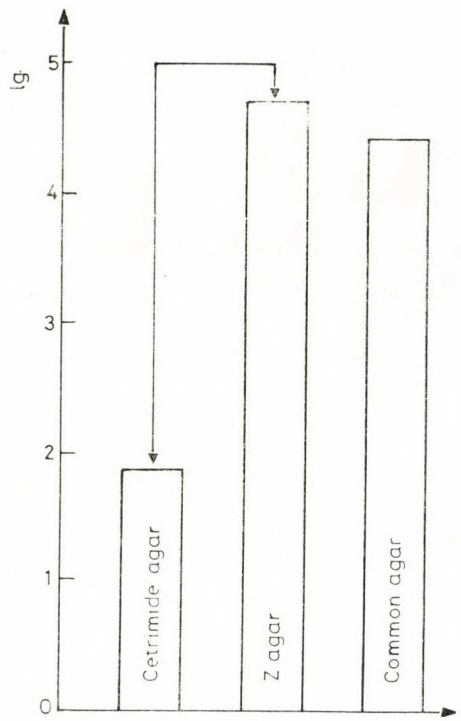

Fig. 2. Microbe counts obtained on cetrimide agar, Z agar and common agar inoculated with dilutions of the broth culture of *Pseudomonas aeruginosa* strain 170 001

ous. The *t* value calculated from the data was 0.068: this value is lower than the lowest value given in Student's table: therefore, the difference is not significant.

The pooled logarithmic mean value obtained for pure cultures inoculated onto cetrimide agar, Z agar and common agar was 1.73, 4.61 and 4.52, respectively. The results are shown in Fig. 2. Between the results obtained on cetrimide agar and Z agar there was a difference of nearly three orders of magnitude; therefore, in that case there was no need for the statistical verification of significance. When comparing Z agar and common agar, the calculated statistical *t* value was 0.43 and the table value was 2.36: thus, the difference was not significant.

The logarithmic values obtained for the raw milk samples are shown in Table I.

The logarithmic mean value obtained for cetrimide agar and Z agar inoculated with raw milk samples was 1.79 and 3.03, respectively (Fig. 3). The methodological source of error was 0.396 and 0.108, and the F value considerably exceeded the table value (13.35 *vs.* 1.84), indicating that the standard deviations were not homogeneous. In that case the *d* test had to be used instead of the two-sample *t* test. The result (9.49) markedly exceeded

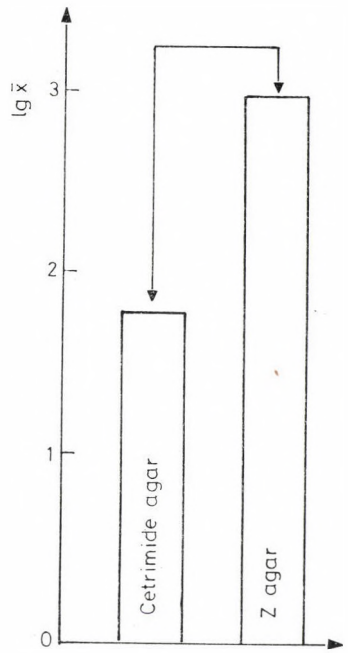

Fig. 3. Pseudomonas aeruginosa counts cultured from raw milk samples on cetrimide agar and Z agar

the corresponding value in Student's *t* table, suggesting a highly significant difference.

Of *Pseudomonas* bacteria inoculated into nitrofurantoin broth, both *P. aeruginosa* and *P. mendocina* grew well, whereas in the Z broth the latter bacterium failed to grow (Table II).

Conclusions

The calculations showed no difference in sensitivity between the nitrofurantoin broth and the Z broth. At the same time, the synthetic broth directly indicates the biochemical properties most important for bacterium identification. Besides the complete nitrate reduction, the medium indicates fluorescein pigment production. There is no need for the oxidase test either, as only bacteria of oxidative properties can utilize acetate as carbon source.

A considerable difference was demonstrable between the cetrimide agar and Z agar in favour of the latter. More colonies grew out on Z agar than on common agar; this difference, however, was not statistically significant.

Z agar was significantly better than cetrimide agar also when raw milk samples were examined. The standard deviation of the results obtained on cetrimide agar was higher.

The plates were examined in ultraviolet light to demonstrate fluorescence of the colonies. By this method the colonies can be demonstrated rather early, as fluorescein diffuses into the medium in an area much larger than the colonies.

The main advantages of culturing *P. aeruginosa* in synthetic media are as follow:

1. Synthetic media do not contain inhibitory substances and, therefore, have higher sensitivity.

2. Using synthetic media, the most important biochemical reactions (nitrate reduction, oxidase reaction and pigment production) serving for the identification of *P. aeruginosa* can be read and evaluated directly, which saves time and medium.

3. The medium consists of simple, chemically well defined components and, thus, is of standard quality.

4. The synthetic medium can be stored for a long time as its components are not labile and enrichment of inhibitory substances need not be reckoned with.

6. The synthetic medium can be produced at a low cost.

7. It can be produced also in powder form.

Owing to these advantages, the new synthetic media described in this paper would be highly suitable for use in food microbiology. Their industrial-scale production would be possible, too.

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GAS CHROMATOGRAPHIC CHARACTERIZATION OF THE RELATIONSHIP BETWEEN SOME *MYCOBACTERIUM AVIUM* AND *MYCOBACTERIUM PARATUBERCULOSIS* STRAINS

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Mycobacterium avium strain P-55 and *M. avium* strain DENT differ from *M. avium* strain 16909-338 on the basis of their fatty acid spectra (C14:0, C18:0 and tuberculostearic [TBS] acids) studied by multivariate statistical analyses. Strains P-55 and DENT are closer to *M. paratuberculosis* strain 5889 than to *M. avium* strain 16909-338, a finding which is in harmony with earlier immunological observations. The recently isolated *M. paratuberculosis* strain 385 has proved different from *M. paratuberculosis* strain 5889.

Keywords: Gas chromatography, characterization, *Mycobacterium avium*, *Mycobacterium paratuberculosis*.

The differentiation of *Mycobacterium avium* and *M. paratuberculosis* strains is based upon mycobactin dependence (Chiodini, 1986; Thoen, personal communication; Thorel, 1984). In biochemical properties there is hardly any difference between *M. paratuberculosis* and *M. avium* strains, except the hydrolysis of Tween 80 and the presence of acidic phosphatase in some strains (Thorel and Valette, 1976), and generally they are not distinguishable even in animal experiments (Andersen et al., 1982; Körmendy et al., 1986).

The complement fixation tests between immune sera produced against *M. avium* strains (Shaefer's method) and paratuberculosis antigens showed that P-55 and DENT antisera gave cross reactions of high titre (Körmendy et al., 1984).

The aim of our investigation was to get to know whether the similarity of strains P-55 and DENT to *M. paratuberculosis* was manifested also in the fatty acid composition. The fatty acid profiles of whole cells correspond to the genetic information contained in the chromosome. The gas chromatographic (GC) analysis of mycobacterial fatty acids is a useful method for taxonomic studies and routine differential diagnosis (Daffé et al., 1983; Larsson, 1983; Ohashi et al., 1977; Thoen et al., 1971; Tisdall et al., 1979).

Materials and methods

Strains

The following strains were used: *Mycobacterium avium* 16909-338 ATCC (*M. avium* 16909-338); *M. avium* P-55 ATCC (P-55); *M. avium* DENT ATCC (DENT); *M. paratuberculosis* 5889 Praha, Bergey 1923 (PTBC 5889); *M. paratuberculosis* 385, isolated from cattle, primary culture on Herold's medium (PTBC 385).

Cultivation

The strains were cultivated on Herold's medium containing 2 mg/1000 ml mycobactin, at 37 °C. The cells were collected after 3 weeks, except PTBC 385 which required 8 weeks cultivation time. Four inoculations were made from each strain. The cells were harvested by gently scraping the surface of the medium with a 4-mm incubating loop.

Saponification, methylation

Miller's method (Miller, 1984) was used with slight modifications.

The cells were suspended in 1.0 ml of 1.2 M NaOH in 50% aqueous methanol solution in a 100 mm × 13 mm screw cap test tube. The tubes were sealed tightly and then placed in a boiling water-bath. After 30 min the tubes were removed from the water-bath and cooled to room temperature. The saponificate was acidified with 0.5 ml of 6 M HCl (pH < 2). One ml of 14% boron trifluoride in methanol reagent (Merck) was added and the tightly sealed tubes were heated in a water-bath at 85 °C. After 5 min the tubes were removed and cooled to room temperature. The fatty acid methyl esters were extracted with 1.0 ml hexane : diethyl ether (1 : 1) for 3 min. The acidified aqueous (bottom) phase was discarded and 3 ml of 0.3 M NaOH solution was added to the organic extract. After mixing, the top (organic) phase was used for GC analysis.

Gas chromatography

A Hewlett-Packard gas chromatograph (model 5890 A) equipped with flame ionization detector and HP 3390 A integrator was used in all experiments. Injection of the fatty acid methyl ester solution was performed splitless onto a HP-1 column (Methyl Silicone Gum, 5 m × 0.5mm × 2.65 μm film thickness). Nitrogen was used as carrier gas. Injector temperature was 250 °C and detector temperature was 300 °C. Column temperature was programmed from 120 to 256 °C, with a temperature increase of 8 °C per min.

Evaluation of the chromatograms was done from the data of seven main peaks. Each value represents the mean of two analyses. Fatty acids were identified by comparison with retention times of a known standard containing saturated and unsaturated fatty acids (Supelco Bacterial Acid Methyl Ester Mixture, 4-5436). The identification of unknown fatty acids for which no standard was available was based on the calculation of the equivalent chain length (ECL) data, according to Miller and Berger (1985).

Data processing

Cluster analyses were computed using single linkage, complete linkage and unweighted group average amalgamation methods (Hartigan, 1975). To eliminate the mutual correlations of the variables a principal component analysis (PCA) was applied (Mardia et al., 1979).

Statistical analyses were done using SPSS+program package rented from the Program Library of the Hungarian Academy of Sciences.

Results and discussion

The fatty acid spectra of the investigated strains were qualitatively similar to one another. However, the percentual distribution of the fatty acids was different (Table I).

First of all, cluster analysis, a method often used in numerical taxonomy, was used to get a raw general picture (Fig. 1). Two main clusters were found. The first one includes strains DENT, P-55 and PTBC 5889, while the second

Table I

Comparison of the percentage of fatty acids in five *Mycobacterium* strains

Carbon no. and no. of C–C double bounds	M. av. 16909–338		P–55		DENT		PTBC 5889		PTBC 385	
	Mean per cent	SD	Mean per cent	SD	Mean per cent	SD	Mean per cent	SD	Mean per cent	SD
14:0	3.09	1.00	3.10	0.45	5.83	0.45	4.68	0.48	0.79	0.31
16:1	4.07	1.60	7.29	0.91	5.88	0.36	3.40	0.26	2.15	1.02
16:0	33.75	3.44	35.02	2.79	37.51	1.73	39.58	1.49	36.85	4.21
17B*	5.94	1.32	5.30	1.38	5.30	0.84	8.45	1.73	4.01	1.34
18:1	26.86	3.25	17.21	2.17	16.31	0.74	11.05	0.60	30.87	4.81
18:0	12.92	3.36	4.66	2.10	7.97	0.83	2.90	1.96	21.28	2.68
TBS**	13.37	2.11	27.42	4.18	21.20	0.61	29.94	1.97	4.06	0.85

* Identified by ECL values as a saturated branched-chain fatty acid of 17 carbon atoms
** Identified by ECL values as tuberculostearic acid
SD: standard deviation

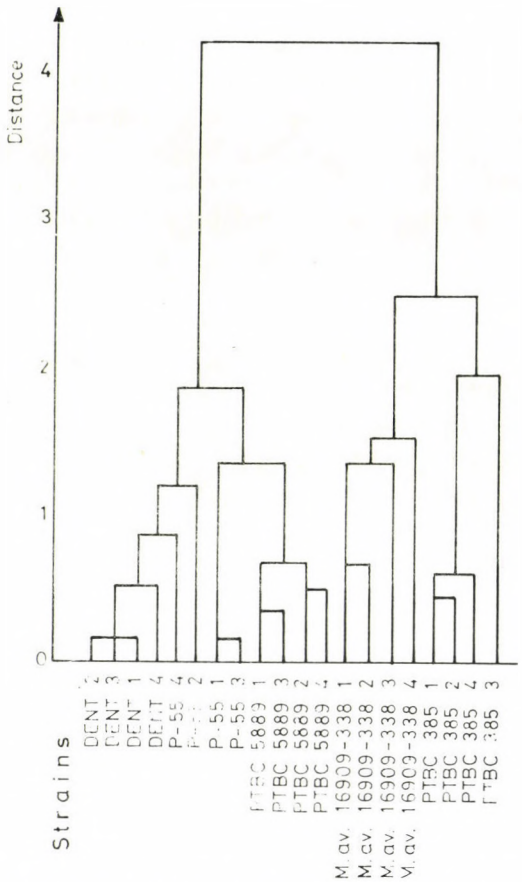

Fig. 1. Cluster analysis of samples computed by complete linkage amalgamation method

one contains *M. avium* strain 16909-338 and PTBC strain 385. At lower level the first main cluster can be divided into two smaller ones: at this level DENT separates completely from PTBC 5889, but P-55 remains partly with DENT, partly with PTBC 5889 and does not form an independent cluster even at lower level. The second main cluster separates at lower level into smaller clusters, i.e. *M. avium* 16909-338 and PTBC 385.

Using PCA, the first two factors cumulatively included 77.9% of the original variance and the first three ones included 90.8%. So one may say that the seven fatty acids are influenced by two or three background factors. On the basis of the factor scores fatty acid 18 : 1 and 18 : 0 form a compact group, and 14 : 0 and tuberculostearic (TBS) acid form another, slacker group which is the stem of a more loose group involving successively the 17B and 16 : 1 fatty acids. If we plot the samples by their factor values, we get a very

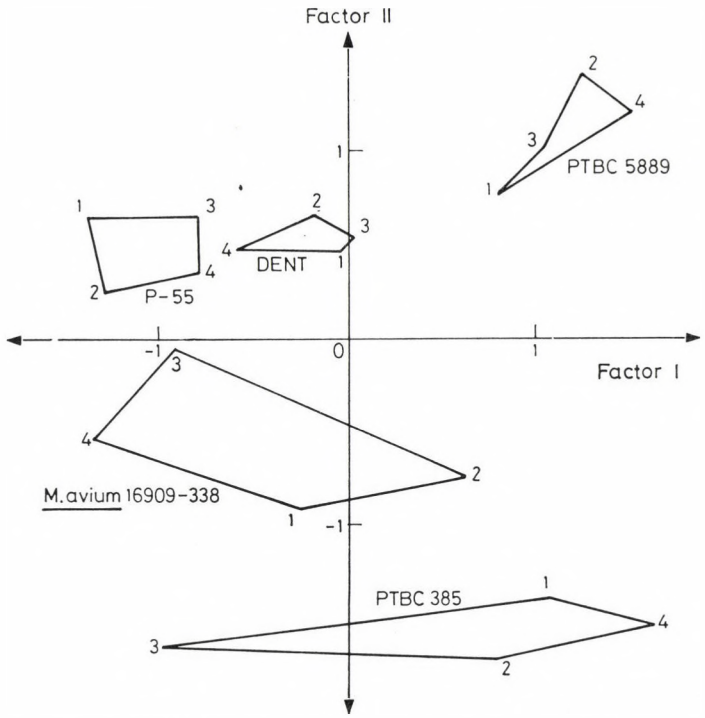

Fig. 2. Strains scatter-plotted by the first and second factors. These two factors contain 77.9% of the original variance

convincing picture (Fig. 2). The strains form non-overlapping groups. In contrast with the results of the cluster analysis even P-55 forms an independent group, being completely separated from both strains DENT and PTBC 5889.

The fatty acids of *M. paratuberculosis* strains were studied by Andersen et al. (1982). These strains had been isolated from goats and sheep and maintained on Middlebrook's 7H10 medium. Their publication does not inform us of strains isolated from cattle. The fatty acid composition of the *M. paratuberculosis* strain isolated from goats contained 14 : 1, 10-ME-16 : 0 and two unidentified constituents eluting in front of 18 : 1, but from the *M. avium*-like strain of sheep origin the 14 : 1 constituent was missing. They found different long-chain fatty acids though in very low quantities. Our results are consistent with this.

Chiodini and Kruiningen (1985) analysed the cellular fatty acid composition of *M. paratuberculosis* of bovine origin. However, they cultured the strain on Herold's egg yolk medium. They formed the opinion that the variability in chromatographic peaks within the same *Mycobacterium* spp. might reflect differences in the length of incubation (degradation of dead organisms), the mycobacterial strain and the growth medium. They did not find differences

among strains of bovine, caprine and ovine origin and did not study the age of the strains. They established that the chromatograms of *M. paratuberculosis* contained 34 of 41 peaks of which 12 peaks were inconsistently present.

As opposed to Chiodini's study (Chiodini, 1986), we isolated *M. paratuberculosis* of S colony morphology in primary culture. This isolate may change into a strain of R colony morphology during maintenance.

Damato et al. (1987) established that the fatty acid spectra of the 36 *M. paratuberculosis* isolates contain fatty acid 14 : A, and this fraction is missing from *M. avium–M. avium-intracellulare* organisms. In contrast to our investigation, they cultured the organisms in Middlebrook 7H12 broth. We did not find the 14 : A peak in our chromatograms in a quantity sufficient for mathematical evaluation.

Saxegaard and Baess (1988) established by DNA-DNA hybridization that there is only marginal genetic difference between *M. avium* and *M. paratuberculosis*. However, the latter authors examined *M. paratuberculosis* and *M. avium* reference strains and wood pigeon strains only. Further taxonomic studies would be needed.

It is remarkable that the two PTBC strains considerably differ from one another. This difference may be due to the different cultivation times (PTBC 385 was a primary culture with a longer cultivation time, in contrast to the museum subculture, PTBC 5889). Variations in incubation time did not affect the fatty acid spectrum of *M. kansasi* (Saxegaard and Baess, 1988), *M. chelonei*, *M. fortuitum* and *M. smegmatis* (Larsson et al., 1985). *M. paratuberculosis* strains have not yet been studied. Supposing that their cultivation behaviour is similar, the difference between the fatty acid spectrum of PTBC 5889 and that of PTBC 385 is due to changes of the PTBC strains during maintenance.

The results of multivariate statistical analyses show that both P-55 and DENT separate from *M. avium* 16909-338. P-55 and DENT are situated nearer to PTBC 5889 than to *M. avium* 16909-338. These results are in good correlation with the observations of Körmendy et al. (1984), namely, that the immunological behaviour of P-55 and DENT differs from that of other *M. avium* strains and shows a similarity to PTBC 5889.

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IMMUNOPATHOLOGICAL CHANGES IN MICE CAUSED BY *BORDETELLA BRONCHISEPTICA* AND *PASTEURELLA MULTOCIDA*

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The immunopathological changes induced by toxigenic *Pasteurella multocida*, toxigenic phase I *Bordetella bronchiseptica* and its phase III variant were studied. Four groups of mice, each containing 21 animals, were inoculated intravenously with sublethal doses of *B. bronchiseptica* as follows: group 1: phase I toxigenic *B. bronchiseptica* whole-cell suspension (WCS); group 2: phase III *B. bronchiseptica* WCS; group 3: phase I *B. bronchiseptica* sonicated extract (SE); and group 4: phase III *B. bronchiseptica* SE. The fifth group received SE of toxigenic *P. multocida*. Lymphatic organs, lungs, livers and testes from three mice per group were examined histologically on every second day of a two-week period.

In the spleen of mice, where the so-called lienotoxic effect manifested itself (groups 1, 3, 4 and 5), the percentile proportion of lymphoblasts significantly decreased in both the B and the T cell dependent areas. The lymph nodes also showed a reduction in the number of lymphoblasts. The reduction in spleen mass was partly attributable to a drastic decrease in the number of megakaryocytes and of blast cells participating in physiological extramedullary haematopoiesis in the red pulp.

Opposite changes were demonstrable in group 2 the mice of which showed splenic hypertrophy.

Keywords: Immunopathological changes, *Pasteurella multocida*, *Bordetella bronchiseptica*, mice.

Bordetella bronchiseptica and *Pasteurella multocida* are involved in the aetiology of atrophic rhinitis (AR) of swine (Pedersen and Barfod, 1981; Rutter and Rojas, 1982). Both species produce a toxic substance which seems to have a crucial role in the pathogenesis of AR (Il'ina and Zasukhin, 1975; Pedersen and Barfod, 1981; Rutter and Rojas, 1982; Chanter et al., 1986; Magyar et al., 1988). These toxins are heat labile and they have a number of similar biological activities. They are lethal in mice (Rutter, 1983; Collings and Rutter, 1985), cytotoxic to embryonic bovine lung (EBL) cells (Rutter and Luther, 1984; Collings and Rutter, 1985), and cause dermonecrosis in the guinea pig skin test (de Jong et al., 1980). Furthermore, they show lienotoxicity in intravenously inoculated mice (Krüger and Horsch, 1982; Magyar, 1989). Although some aspects of this phenomenon have recently been investigated (Magyar, 1989 and 1990), our knowledge of the histopathological lesions caused by these organisms in mice is rather limited.

The aim of the present study was (*i*) to investigate and compare the immunopathological changes caused by toxigenic *P. multocida*, toxigenic phase I *B. bronchiseptica* and its phase III variant, and (*ii*) to gain data about the pathogenesis of these changes.

Materials and methods

Mice

Inbred albino mice of strain CFLP (LATI, Gödöllő) of both sexes, 16–18 g in body mass, were used.

Bacterial strains

B. bronchiseptica CF was isolated in a pig herd with clinical signs of AR. This strain was haemolytic, produced bovine haemagglutinin (Semjén and Magyar, 1985) and adenylate cyclase enzyme, and its cell-free sonicated extract was cytotoxic to EBL cells so it was regarded as a toxigenic phase I strain. *B. bronchiseptica* CFp61 was a phase III variant of strain CF obtained by repeated subculturing on MacConkey agar plates. It had lost its haemolysin, bovine haemagglutinin and adenylate cyclase enzyme activity but its sonicated extract (SE) retained some cytotoxic activity (Magyar, 1990).

P. multocida P108 (capsule type D) was isolated in the same herd and its extract was highly cytotoxic (Magyar, 1989).

Preparation of whole-cell suspensions and cell-free sonicated extracts

This was done as described previously (Magyar, 1990).

Experimental design

The design of the experiment is shown in Table I. The degrees of dilutions needed for getting sublethal doses were determined by the lienotoxicity test using twofold serial dilutions. Five groups, each containing 21 mice, were inoculated intravenously. Each mouse received 0.2 ml of the test material.

Necropsy and histology

Three mice of each group were euthanatized on every second day starting from postinoculation (PI) day 1 until PI day 13. The spleen masses were determined and relative spleen values were calculated (spleen mass (mg) per body mass (g) $\times$ 10).

Table I

Experimental design

Group*	Strain	Inoculum	Dilution
1	*Bordetella bronchiseptica* CY	WCS	1:32
2	*Bordetella bronchiseptica* CF_p61	WCS	UD
3	*Bordetella bronchiseptica* CF	SE	1:32
4	*Bordetella bronchiseptica* CF_p61	SE	1:4
5	*Pasteurella multocida* Pl08	SE	1:64

WCS = whole-cell suspension; SE = cell-free sonicated extract; UD = undiluted;
* each group consisted of 21 mice

The spleen, the thymus, the prepatellar and mesenteric lymph nodes, the liver, the lungs and one of the testes were fixed in 5% formalin buffered with NaH_2PO_4 and NaOH (pH 7.2), then embedded in paraffin. The sections were stained with haematoxylin and eosin. For more detailed study of certain leucocyte types the Giemsa stain and the periodic acid–Schiff (PAS) reaction complemented with diastase digestion were used.

Electron microscopy

The tissue samples were fixed in 2.5% glutaraldehyde solution buffered with sodium cacodylate, post-fixed in 2% osmium tetroxide, then the organ pieces were embedded in Durcupan ACM resin. Subsequently the samples were sectioned with a Reichert-OMU-3 ultramicrotome, counterstained with uranyl acetate and lead citrate, and examined in a Philips 201 CS electron microscope.

Results

Lienotoxicity

The WCS and SE of *B. bronchiseptica* CF (groups 1 and 3) as well as the SEs of *B. bronchiseptica* CFp61 and *P. multocida* P108 (groups 4 and 5) caused atrophy of the spleen, while the WSC of *B. bronchiseptica* CFp61 induced remarkable spleen hypertrophy. The changes in relative spleen masses are shown in Fig. 1.

Histology and electron microscopy

In the *spleen* of *group 1* mice, from PI day 2 the ratio of lymphoblasts, as compared to that of differentiated lymphocytes and plasma cells, gradually decreased both in the germinative centres of the Malpighian bodies (B cell

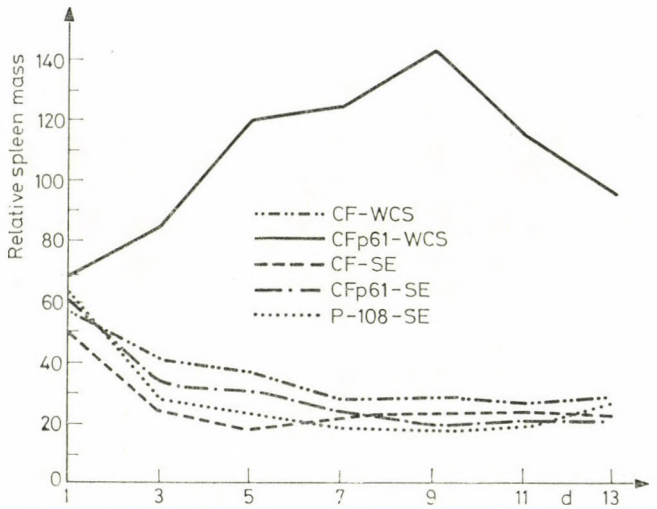

Fig. 1. Changes in relative spleen mass in experimental groups 1–5 inoculated with whole-cell suspensions (WCS) of *Bordetella bronchiseptica* strain CF and CFp61 and the cell-free sonicated extracts (SE) of *B. bronchiseptica* CF, CFp61 and *Pasteurella multocida* strain P108. All values represent the mean calculated for 3 mice per group. The control value is 55

dependent zones) and in the periarteriolar lymphoid sheath (T cell dependent zones) (Table II). The lymphoblasts were characterized by a pale nucleus that had a less electron dense chromatin substance than that of the differentiated lymphocytes. Their cytoplasm was larger and richer in cell organelles. In the B and T cell dependent zones the ratio of lymphoblasts decreased from 90% and 40%, respectively, to about 5%. This was accompanied by a proportional decrease in the diameter of the Malpighian bodies (to one-third or one-fifth of the control). Up to PI day 8 the depletion of differentiated lymphocytes was not expressed in these areas; however, on PI days 8–14 it was pronounced in the germinative centres. In cell colonies of extramedullary haematopoiesis in the red pulp the ratio of undifferentiated blast cells and the number of megakaryocytes occurring in singles gradually decreased from PI day 2, and these cells were already absent between PI days 10 and 14 (Figs 2—7).

After the inoculation the ratio of lymphoblasts, as compared to that of differentiated lymphocytes and plasma cells, decreased gradually also in the *lymph nodes* of *group* 1 mice, both in the germinative centres of the cortex (B cell dependent zones) and in the paracortical region (T cell dependent zone). On PI days 10 through 14 a mild depletion was observed. From PI day 6, the cortex of the *thymus* narrowed down and became depleted in lymphocytes as compared to the control. In the majority of group 1 mice the *liver* cells belonging to the reticulo-histiocyte system (RHS) became activated and in some areas showed focal proliferation. In addition, inflammatory-necrotic foci

Table II

Immunopathological changes in the spleens of mice inoculated with whole-cell suspensions or cell-free sonicated extracts of *B. bronchiseptica* or cell-free sonicated extract of *P. multocida*

Immunopathological changes		Group 1 (CF WCS) PI day 1	3	5	7	9	11	13
Lymphoblast % in malpighian bodies	T dependent zone	40	30	20	10	10	5	5
	B dependent zone	90	70	40	20	10	10	5
Depletion of lymphocytes	T dependent zone	+	+	+	+	+	+	+
	B dependent zone	+	+	++	++	++	++	++
Numbers of haematopoetic cell colonies in the red pulp□		12	10	7	5	2	0	0

Immunopathological changes		Group 2 (CFp61 WCS) PI day 1	3	5	7	9	11	13
Lymphoblast % in malpighian bodies	T dependent zone	40	40	50	70	70	80	80
	B dependent zone	90	90	95	95	95	98	98
Depletion of lymphocytes	T dependent zone							
	B dependent zone							
Numbers of haematopoetic cell colonies in the red pulp□		12	14	14	16	16	16	16

Immunopathological changes		Control
Lymphoblast % in malpighian bodies	T dependent zone	40
	B dependent zone	90
Depletion of lymphocytes	T dependent zone	
	B depeudent zone	
Numbers of haematopoetic cell colonies in the red pulp□		12

Immuno pathological changes		Group 3 (CF SE) PI day 1	3	5	7	9	11	13
Lymphoblast % in malpighian bodies	T dependent zone	40	25	20	10	10	5	5
	B dependent zone	90	65	35	30	20	10	5
Depletion of lymphocytes	T dependent zone	+	+	+	+	+	+	+
	B dependent zone	+	+	+	++	++	++	++
Numbers of haematopoetic cell colonies in the red pulp□		11	8	6	4	2	0	0

* weak: ** remarkable: □ means for 10 visual fields at a magnification of × 400

Table II continued

Immunopathological changes		Group 4 (CFp61 SE) PI day 1	3	5	7	9	11	13
Lymphoblast % in malphigian bodies	T dependent zone	40	40	30	20	10	10	10
	B dependent zone	90	90	80	70	50	30	20
Depletion of lymphocytes	T dependent zone	+	+	+	+	+	+	+
	B dependent zone	+	+	+	+	++	++	++
Numbers of haematopoetic cell colonies in the red pulp□		12	10	8	6	2	0	0

Immunopathological changes		Group 5 (P 108 SE) PI day 1	3	5	7	9	11	13
Lymphoblast % in malpighian bodies	T dependent zone	40	25	20	10	10	5	5
	B dependent zone	90	65	35	30	20	10	5
Depletion of lymphocytes	T dependent zone	+	+	+	+	+	+	+
	B dependent zone	+	+	+	++	++	++	++
Numbers of haematopoetic cell colonies in the red pulp		11	8	6	4	1	0	0

* weak; ** remarkable; □ means for 10 visual fields at a magnification of ×400

containing lymphocytes and granulocytes (Fig. 8) were seen dispersed in the parenchyma. In the *lungs*, the walls and lumina of some major respiratory passages and the surrounding alveoli were infiltrated by granulocytes, detached epithelial cells and fibrin (Fig. 9). The *testis* was histologically normal and indicated active spermatogenesis.

In the *spleen* of *group 2* mice, after inoculation the ratio of lymphoblasts, as compared to that of differentiated lymphocytes and plasma cells, gradually increased both in the germinative centres of the Malpighian bodies and in the periarteriolar areas. Parallel to this, the diameter of the Malpighian bodies also increased. In the red pulp, in cell colonies of extramedullary haematopoiesis the ratio of undifferentiated blast cells rose as well as the number of megakaryocytes. There was lymphoblast proliferation in the cortex and paracortical zones of the *lymph nodes*, in both zones with the development, and increase in number, of secondary follicles. The histological findings seen in the *thymus* and the *lungs* resembled those found in the controls. The *liver* of some mice contained, here and there, a few megakaryocytes and cell colonies consisting of normoblasts, normocytes, promyelocytes and metamyelocytes, indicative of haematopoiesis (Fig. 10).

Both in type and severity, the lesions seen in the *spleen*, *lymph nodes* and *thymus* of *group 3*, *group 4* and *group 5* mice resembled those seen in group 1 animals. However, histologically the *lungs* and the *liver* did not differ from the control.

The examined organs from mice of *group C (untreated control)* were histologically normal.

Discussion

It is known that the heat-labile toxins of *B. bronchiseptica* and *P. multocida* induce remarkable spleen mass reduction in mice intravenously inoculated with them at sublethal doses (Krüger and Horsch, 1982; Magyar, 1989). Éliás et al. (1983) studied the lienotoxic effect of *B. bronchiseptica* live cultures and attributed it to lymphocyte depletion and degenerative changes of lymphocytes (karyopycnosis and karyorrhexis). The results shown here indicate, however, that atrophy of the lymphatic organs is mainly due to a reduction in, or cessation of, the blastogenic transformation of lymphocytes, i.e. to the fact that the formation of these cells does not take place. A similar phenomenon can be observed for extramedullary haematopoiesis in the red pulp of the spleen, which also contributes to the reduction in spleen mass. Lymphocyte depletion and necrosis seem to be of secondary importance in the pathogenesis of the lienotoxic effect. These changes are much more expressed after the administration of other substances, e.g. immunosuppressive trichothecene mycotoxins (Glávits and Ványi, 1988).

The present results indicate that the lienotoxic effect of *B. bronchiseptica* and *P. multocida* heat-labile toxins is essentially a selective influence exerted on the multiplication of cells belonging to the lymphoid, myeloid and platelet system. These toxins are not likely to have a general inhibitory effect on mitosis, as spermatogenesis, i.e. mitosis of the germ cells, remained undisturbed in the testes. Both virulent live *B. bronchiseptica* and the crude extracts of virulent and avirulent *B. bronchiseptica* exerted the effect characterized above. Furthermore, the cell-free sonicated extract of the *P. multocida* strain tested, also causing spleen mass reduction, produced very similar changes. In this respect no difference was demonstrable between the heat-labile toxins of the two bacteria, which is a new contribution to resemblances existing between the two species in biological activities. Perhaps this can be attributed to the fact that these cell populations react to similar (but not wholly identical) stimuli in the same way, in this case with reduced multiplication of the cells.

The live phase III *B. bronchiseptica* strain, though maintained part of its toxigenicity, induced stimulation of the immune system by markedly enhancing blastogenic transformation of both the lymphocytes and the cell colonies of extramedullary haematopoiesis. This may suggest that this low toxigenicity is

not sufficient for producing a lienotoxic effect. It is questionable whether this phase III strain of low toxigenicity can produce antitoxic immunity.

Through their effect exerted on lymphocyte proliferation, *B. bronchiseptica* and *P. multocida* probably can alter both the humoral and the cell-mediated immune response. Their influence on the multiplication of cells belonging to the myeloid and platelet system raises the possibility that they can exert an adverse effect on processes (inflammatory reaction, blood coagulation, etc.) associated with these systems. These questions should be the subject of future investigations.

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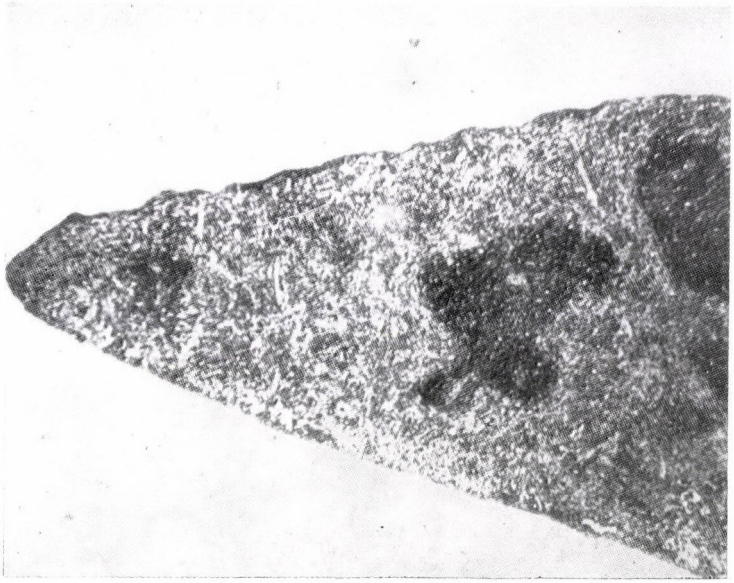

Fig. 2. Spleen of reduced size (weighing 30 g) from a mouse inoculated with *Bordetella bronchiseptica* strain CF and killed on postinoculation (PI) day 13. The Malpighian bodies are small and composed of dark staining cells. The red pulp is deficient in cells. Haematoxylin and eosin. (H. and E.), ×35

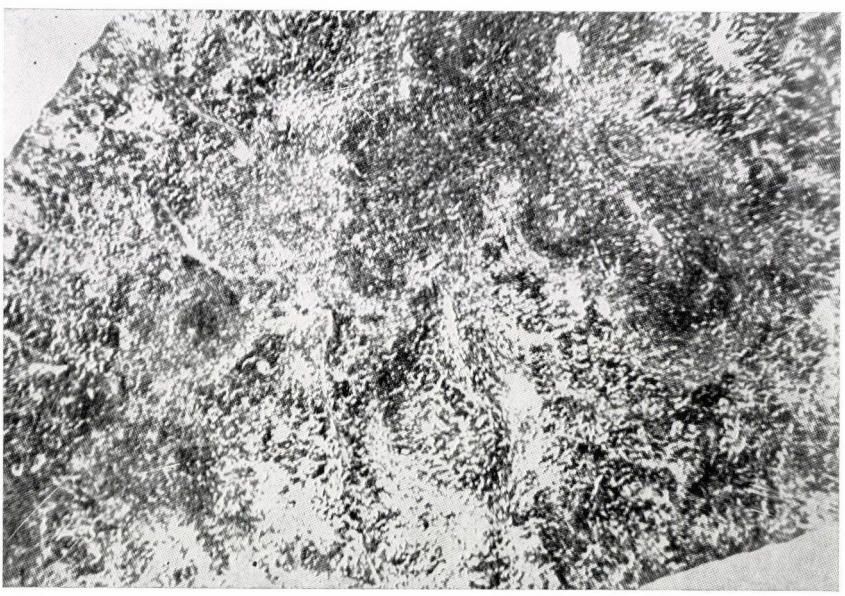

Fig. 3. Enlarged spleen (weighing 120 g) from a mouse inoculated with *Bordetella bronchiseptica* CFp61 and killed on PI day 13. The Malpighian bodies are large and composed of light staining cells. The red pulp is rich in cells. H. and E., ×35

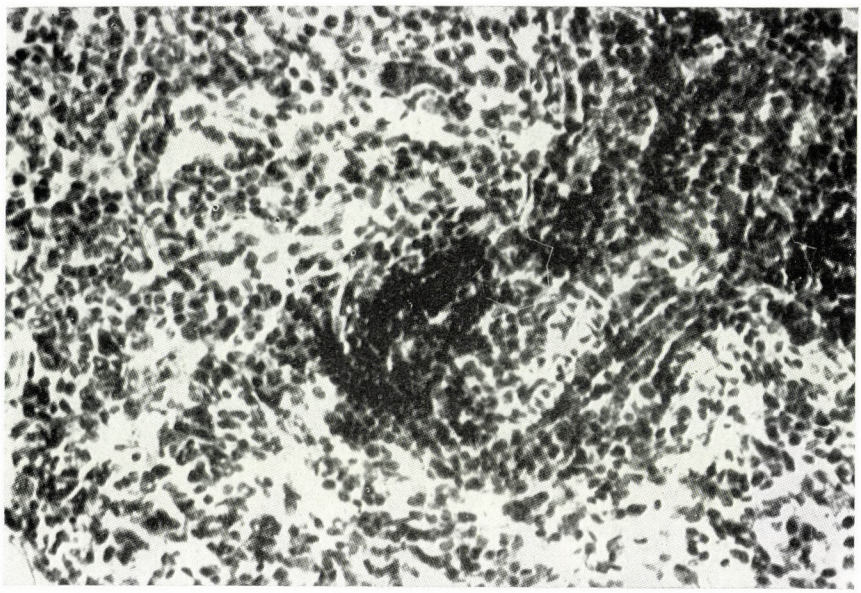

Fig. 4. Detail of Fig. 2. The small Malpighian body is composed of dark staining, differentiated lymphocytes. The red pulp contains differentiated cells. H. and E., ×160

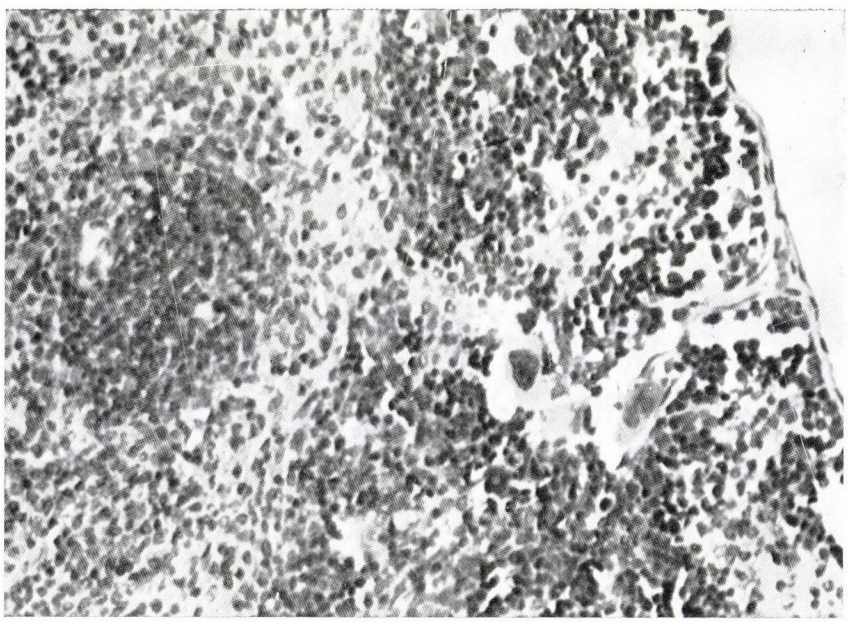

Fig. 5. Detail of Fig. 3. The large Malpighian body is composed of light staining, undifferentiated lymphoblasts. The red pulp contains mostly undifferentiated blast cells and megakaryocytes. H. and E., ×160

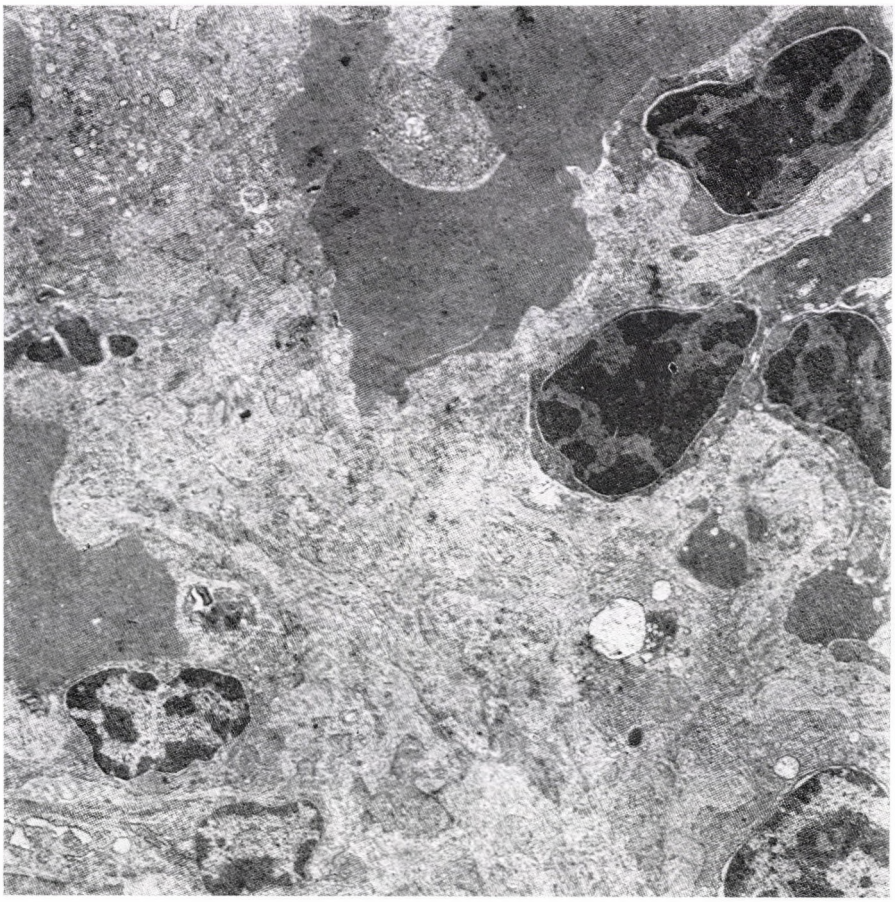

Fig. 6. Spleen of reduced size from a mouse inoculated with *Bordetella bronchiseptica* strain CF and killed on PI day 13. Note the few mature, differentiated lymphocytes in the Malpighian body. Electron micrograph, ×6,400

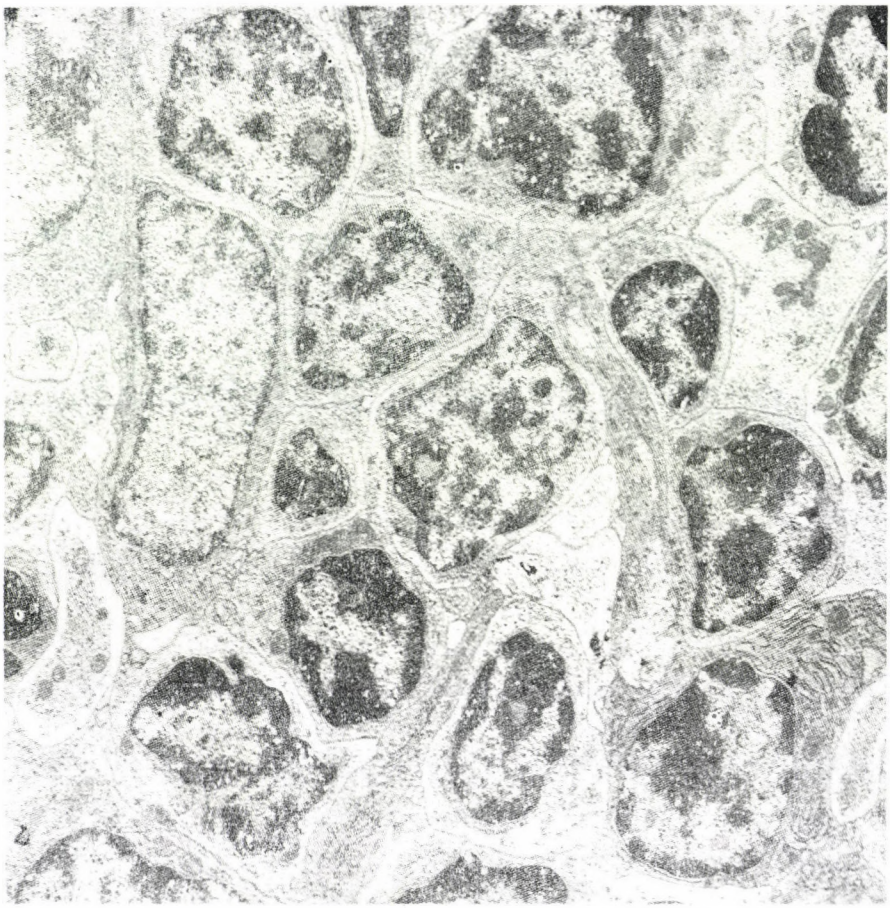

Fig. 7. Enlarged spleen from a mouse inoculated with *Bordetella bronchiseptica* strain CFp61 and killed on PI day 13. Note the large numbers of immature, undifferentiated lymphoblasts in the Malpighian body. Electron micrograph, $\times 6{,}400$

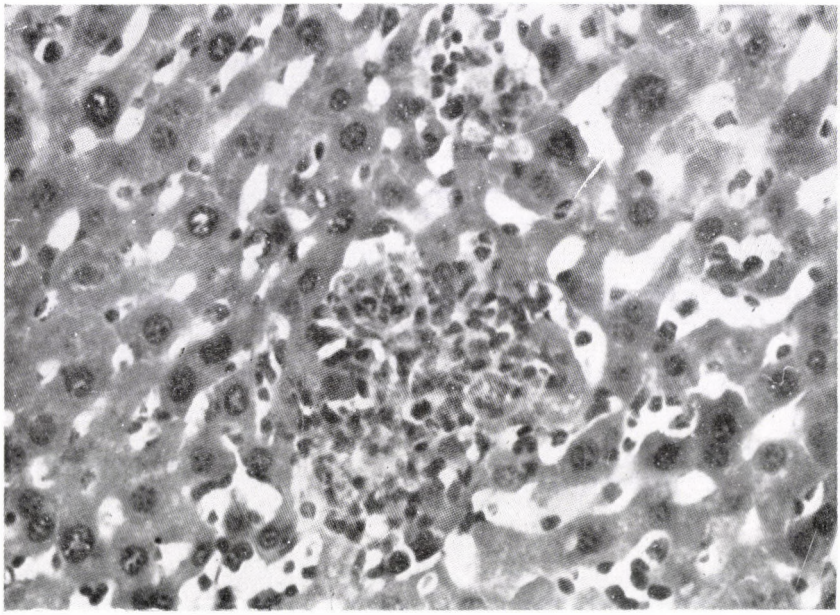

Fig. 8. Inflammatory-necrotic focus in the liver from a mouse inoculated with *Bordetella bronchiseptica* strain CF and killed on PI day 11. H. and E., ×240

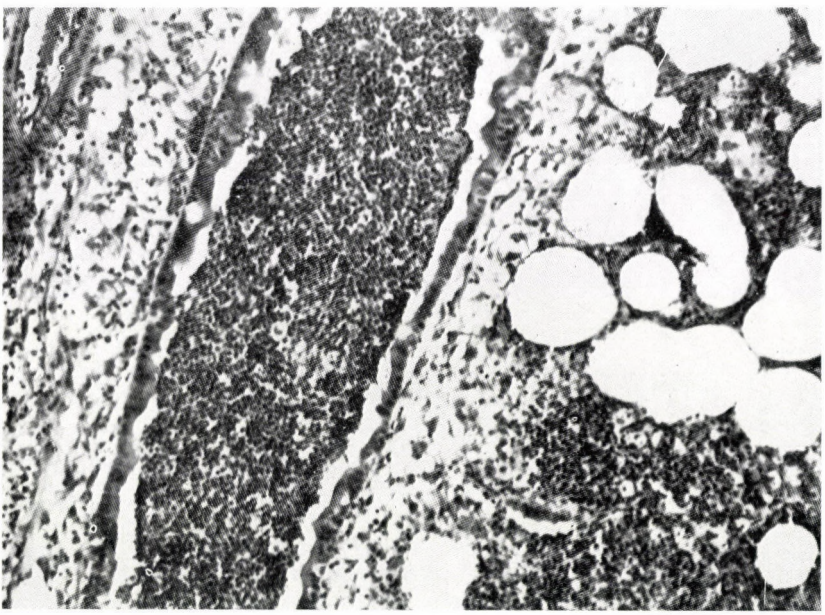

Fig. 9. Catarrhal-purulent bronchopneumonia in a mouse inoculated with *Bordetella bronchiseptica* strain CF and killed on PI day 11. H. and E., ×160

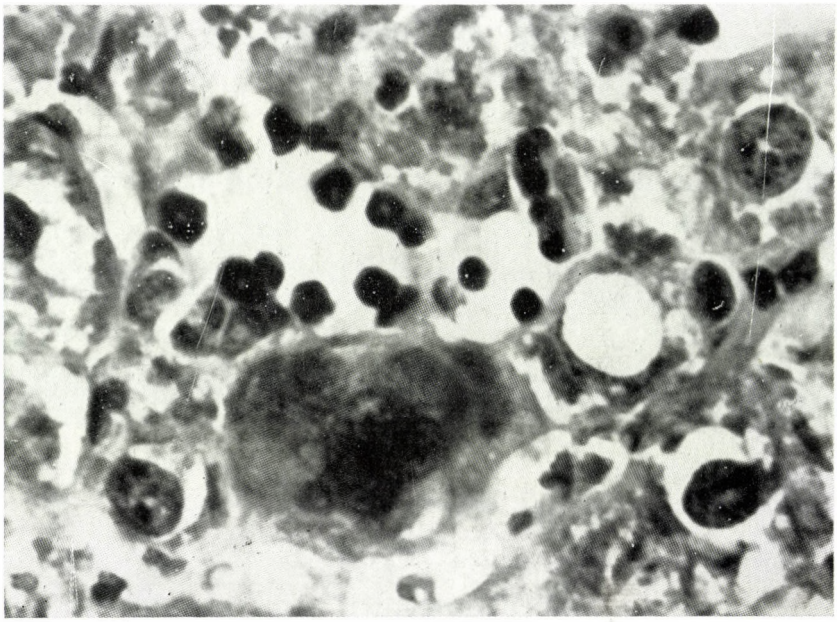

Fig. 10. Haematopoietic cell colony and megakaryocyte in the liver from a mouse inoculated with *Bordetella bronchiseptica* strain CFp61 and killed on PI day 11. H. and E., ×400

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THE PATHOLOGY OF EXPERIMENTAL RESPIRATORY INFECTION WITH *PASTEURELLA MULTOCIDA* AND *BORDETELLA BRONCHISEPTICA* IN RABBITS

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Groups of female New Zealand White rabbits, 8–10 weeks old, were inoculated intranasally with three different *Pasteurella multocida* serotypes (A:3, A:4 and A:12) or one of three *Bordetella bronchiseptica* strains of rabbit origin. Seven out of 18 rabbits died of experimental infection with *P. multocida*. *B. bronchiseptica* killed 3 out of the 8 animals inoculated with it. Deaths occurred between 3 and 6 days postinoculation (PI).

In the rabbits that died of *P. multocida* inoculation, necropsy and histology revealed severe pleuritis with the accumulation of a remarkable amount of fibrinopurulent exudate in the thoracic cavity, serous rhinitis and tracheitis, acute hepatitis with necrotic foci in the parenchyma, and atrophy of the lymphoid organs and tissues. Rabbits killed 10 days PI developed only subacute serous rhinitis and hyperplasia of the lymphoid tissues.

Rabbits that died of *B. bronchiseptica* inoculation showed acute serous rhinitis, acute catarrhal-fibrinopurulent pneumonia and mild pleuritis. As opposed to *P. multocida* inoculated animals, hepatitis and atrophy of the lymphoid tissues were not characteristic of these rabbits. Rabbits killed 10 days PI developed subacute purulent and necrotic pneumonia with remarkable macrophage proliferation, involving all lobes, and hyperplasia of the lymphoid tissues.

Keywords: Pathology, *Pasteurella multocida*, *Bordetella bronchiseptica*, rabbits.

Respiratory diseases occur frequently and cause huge economic losses in domesticated rabbits. The respiratory disease complex may vary from a mild, chronic mucopurulent upper respiratory infection (snuffles) to a more acute or subacute bronchopneumonia (enzootic pneumonia) leading to high mortality (Flatt, 1974). The organisms isolated from these lesions are *P. multocida* and *B. bronchiseptica* (Flatt, 1974; Winsser, 1960).

Pasteurellosis is considered one of the most important diseases of rabbits (Flatt, 1974) which may manifest itself in rhinitis, turbinate atrophy, conjunctivitis, abscessation, otitis media, bronchopneumonia, mastitis, reproductive tract infections and fatal septicaemia (DiGiacomo et al., 1983; DiGiacomo et al., 1989; Percy et al., 1988).

The role of *B. bronchiseptica* has not been satisfactorily elucidated yet. It may be involved in inducing chronic rhinitis and bronchopneumonia (Hagen, 1959; Winsser, 1960). Watson et al. (1975) inoculated rabbits intranasally with either *P. multocida* or *B. bronchiseptica*. The rabbits developed exudative

rhinitis (snuffles) and mild bronchopneumonia. The gross and microscopic lesions were of the same character and distribution in both groups.

The aim of the present work was to study the pathological changes induced by experimental *P. multocida* or *B. bronchiseptica* infection in rabbits.

Materials and methods

Preparation of inocula and reisolation of P. multocida and B. bronchiseptica

P. multocida P68 (A : 4) and P79 (A : 3) were isolated from the nostrils of rabbits exhibiting purulent rhinorrhoea. Strain P18 (A : 12) was isolated from the bone marrow of a rabbit that died of septicaemia. Capsular type was determined by staphylococcal hyaluronidase decapsulation (Carter and Rundell, 1975). Somatic antigens were typed by the gel diffusion precipitin test using rooster antisera described by Heddleston et al. (1972). All the three *B. bronchiseptica* strains (5004, 5023 and 5045) were isolated from the nasal cavities of conventional rabbits. The isolates were identified by standard biochemical tests. *P. multocida* strains were grown from lyophilized stock cultures and inoculated intraperitoneally into mice. The *P. multocida* strains were reisolated from the heart blood of infected mice. *B. bronchiseptica* strains were inoculated onto Bordet-Gengou agar supplemented with 15% sheep blood from the stock cultures, passaged twice on the same medium, and haemolytic phase I colonies were selected for further work. For preparation of inocula for rabbits *P. multocida* strains were propagated on 5% sheep blood agar supplemented with 0.5% yeast extract, and *B. bronchiseptica* on BG agar supplemented with 15% sheep blood. Whole-cell suspensions were prepared in phosphate buffered saline (pH 7.2) and the bacterial suspensions were adjusted to approximately 10^{11} cfu ml^{-1} for *P. multocida* and 5×10^{10} cfu ml^{-1} for *B. bronchiseptica* inocula. Reisolation of *P. multocida* and *B. bronchiseptica* was attempted at necropsy, when samples were taken aseptically from the nasal cavity, lungs and heart blood.

Inoculation of animals

New Zealand female white rabbits, 8–10 weeks old, were used in this study. Before inoculation, the nasal cavity of each rabbit was cultured for *P. multocida* and *B. bronchiseptica*. Rabbits that proved to be free of these microorganisms were inoculated intranasally with 0.5 ml bacterial suspension into each nostril. In the case of *P. multocida* three dilutions of each strain were used (10^{11}, 10^{10} and 10^{9} cfu ml^{-1}), and two animals were inoculated with each

dose. Four rabbits were inoculated with *B. bronchiseptica* strain 5004 and two rabbits with either strain 5023 or strain 5045. Only the undiluted suspensions were used in this case.

Necropsy procedures

All rabbits that died or were euthanatized 10 days PI were examined for gross, and 19 for gross and microscopic lesions (Table I). Tissue samples were collected from the nasal cavity, trachea, lungs, liver, spleen and mesenteric lymph node for histologic examination. The samples were fixed in 5% neutral buffered formalin, then embedded in paraffin. The sections were stained with haematoxylin and eosin.

The nasal cavity, lungs and liver were examined bacteriologically for the presence of *P. multocida* and *B. bronchiseptica.*

Results

Seven out of the 18 rabbits died of *P. multocida* inoculation. *P. multocida* strain P18 proved to be the most lethal, causing fatal disease in 4 out of the 6 rabbits inoculated. *P. multocida* P68 killed two while strain P79 only one rabbit. *B. bronchiseptica* inoculation killed 3 out of the 8 rabbits. Only strain 5004 was lethal. Deaths occurred between PI day 3 and 6 whether inoculated with *P. multocida* or *B. bronchiseptica.*

The pathological changes are summarized in Table I. In rabbits that died of *P. multocida* inoculation, serous fibrinopurulent pleuritis with the accumulation of a large amount of exudate (Figs 1 and 2), serous rhinitis and tracheitis, mild catarrhal and croupous pneumonia, and hepatitis accompanied with the formation of necrotic foci in the parenchyma (Fig. 3) were seen. Atrophy (lymphocyte depletion) was detected in the lymphoid organs and tissues of these animals. In surviving rabbits that were killed 10 days PI, subacute serous rhinitis accompanied with the hyperplasia of lymphoid tissues was found.

Rabbits that died after inoculation with *B. bronchiseptica* developed severe acute catarrhal and croupous pneumonia accompanied with haemorrhage (Fig. 4), acute serous rhinotracheitis (Fig. 5) and mild pleuritis, while hepatitis or atrophy of the lymphoid tissues was not seen in these rabbits. Animals killed 10 days PI developed subacute purulent and necrotic pneumonia (Fig. 6) involving all lobes and characterized by remarkable macrophage proliferation and hyperplasia of the lymphoid tissues.

The *B. bronchiseptica* and *P. multocida* strain used for inoculation were consistently reisolated from the nasal cavities and lungs. Moreover, *P. multocida* could be reisolated also from the liver of rabbits that died of inoculation with this pathogen.

Table I

Pathological changes in rabbits inoculated with *Pasteurella multocida* or *Bordetella bronchiseptica*

Lesion	*Pasteurella multocida*											*Bordetella bronchiseptica*										
	P18			P68			P79	P18		P68	P79	5004			5004		5023		5045			
	10^{11}	10^{11}	10^{10}	10^{9}	10^{11}	10^{10}	10^{11}	10^{10}	10^{9}	10^{10}	10^{11}	5×10^{10}	5×10^{10}	5×10^{10}	5×10^{10}	5×10^{10}	5×10^{10}	5×10^{10}	5×10^{10}	5×10^{10}	5×10^{10}	5×10^{10}
	In rabbits that died						In survivors					In rabbits that died			In survivors							
Serous fibrinopurulent pleuritis	3	3	3	3	3	3	3					1	1	1								
Serous rhinitis	2	2	2	2	2	2	2	1	1	1	1	2	2	1								
Serous tracheitis	2	2	2	2	2	2	2					1	1	1								
Serous hepatitis with necrotic foci	3	1	3	1	3	3	3															
Catarrhal-croupous pneumonia	1	1	1	1	1	1	1					3	2	3								
Subacute purulent-necrotic pneumonia											1				2	3	3	2	3			
Atrophy of the lymphoid tissues (lymphocyte depletion)	2	2	2	1	2	2	2															
Hyperplasia of the lymphoid tissues (lymphoblast proliferation)								1	1	1	1				1	1	1	1	1			

1 = mild lesion; 2 = moderate lesion; 3 = severe lesion

Discussion

Pathological changes similar to those described here are frequently detected in rabbits necropsied in diagnostic institutes. However, Watson et al. (1975) could only find exudative rhinitis and focal bronchopneumonia in rabbits experimentally infected with *P. multocida* or *B. bronchiseptica*. Neither clinical signs of the disease nor deaths occurred during the 3-week observation period. They concluded that it is not possible to differentiate infection produced by either organism based upon the clinical signs, gross lesions or histologic findings.

On the other hand, the results described here suggest that *P. multocida* and *B. bronchiseptica* cause pathomorphological changes that differ from each other in characteristics, distribution and location. Furthermore, similarly to *P. multocida*, *B. bronchiseptica* can also induce fatal respiratory disease in rabbits.

Fig. 1. Lungs from a rabbit that died 4 days after inoculation with 10^{10} cfu of *Pasteurella multocida*. In the markedly widened visceral pleura note a demarcation zone consisting of inflammatory cells. On the surface of the serosa there is inflammatory exudate. Haematoxylin and eosin (H. and E.), $\times 35$

Fig. 2. Detail of Fig. 1. From bottom to top: the pulmonary pleura is oedematous; it shows zonal infiltration with inflammatory cells; and on its surface there is exudate consisting of cell debris and fibrin. H. and E., ×63

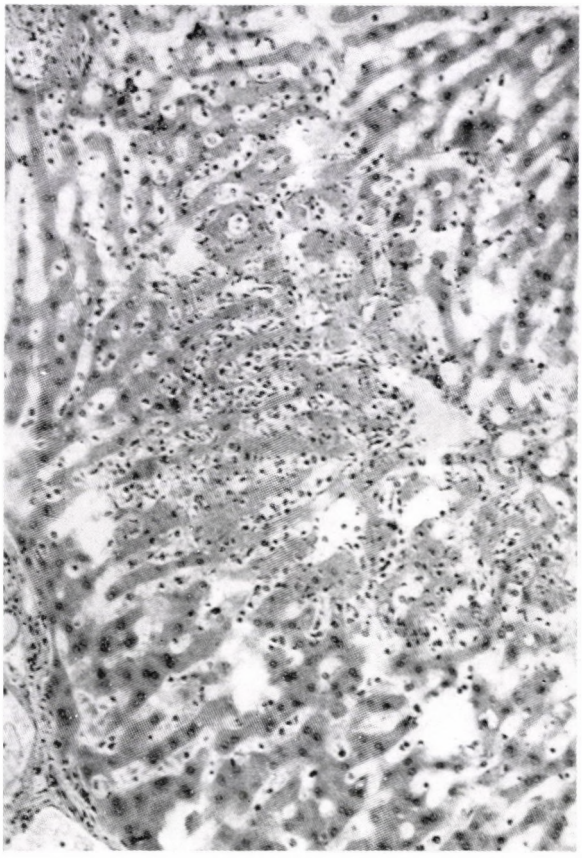

Fig. 3. Acute inflammatory-necrotic focus in the liver from a rabbit that died 3 days after inoculation with 10^{11} cfu of *Pasteurella multocida*. H. and E., $\times 63$

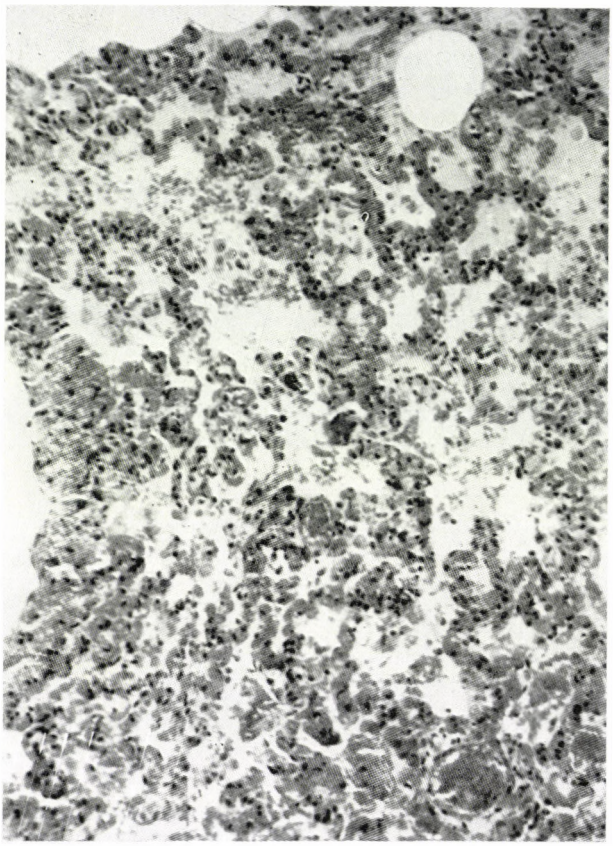

Fig. 4. Lungs from a rabbit that died 5 days after inoculation with 5×10^{10} cfu of *Bordetella bronchiseptica*. Acute catarrhal-croupous pneumonia. H. and E., $\times 63$

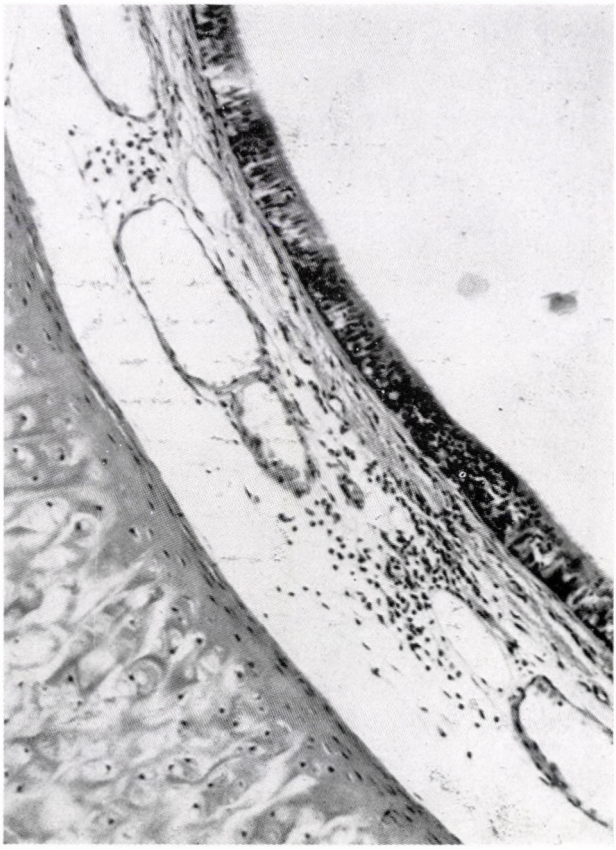

Fig. 5. Acute serous inflammation in the trachea from a rabbit that died 5 days after inoculation with 5×10^{10} cfu of *Bordetella bronchiseptica*. H. and E., $\times 63$

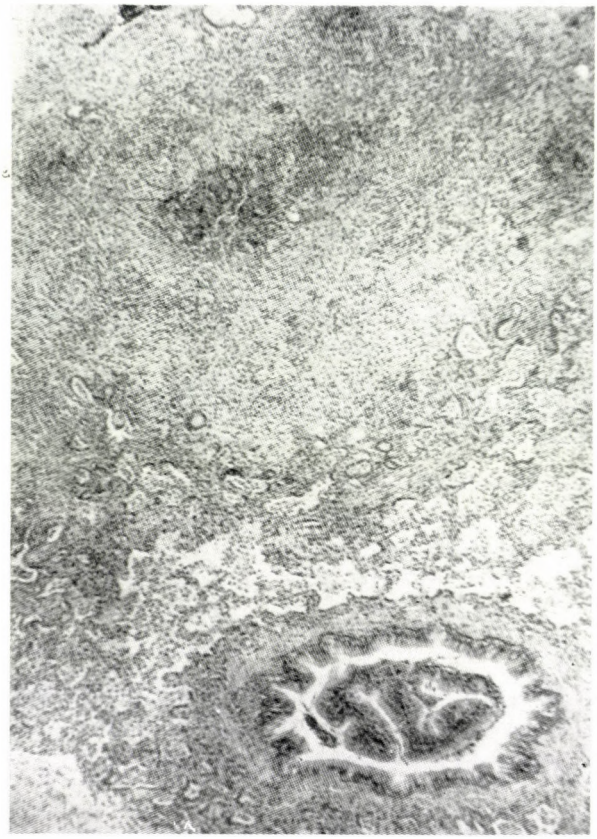

Fig. 6. Lungs from a rabbit euthanatized 10 days after inoculation with 5×10^{10} cfu of *Bordetella bronchiseptica*. Subacute purulent-necrotic pneumonia. H. and E., $\times 35$

Big differences are known to exist in the virulence of various *P. multocida* isolates of rabbit origin (Okerman et al., 1979). Some strains cause fatal septicaemia while others induce the chronic form of rabbit pasteurellosis (snuffles) only. Perhaps the *P. multocida* strain used by Watson et al. (1975) was a less virulent one. The susceptibility of rabbits may also differ. Sometimes it was not possible to reproduce the disease when older rabbits were used (data not shown).

Percy et al. (1988) suggested to consider *B. bronchiseptica* as a potential co-pathogen of the rabbit respiratory tract. The results reported her show that *B. bronchiseptica* can cause severe disease in rabbits also when applied alone. However, further studies are needed to elucidate the potential virulence determinants of this microorganism that may have a role in the pathogenesis of this disease.

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CHANGES IN THE SERUM LEVELS OF PRIMARY BILE ACIDS IN DAIRY COWS

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Serum cholic acid (SCA) and serum chenodeoxycholic acid (SCDCA) concentrations were determined in healthy dairy cows by radioimmunoassay (RIA). The levels of these two primary bile acids were correlated with the cows' reproductive status. The lowest concentrations were measured in dry cows (SCA: 7.8 $\pm$ 3.3 μmol/l, SCDCA.: 1.5 $\pm$ 1.0 μmol/l). In freshly calved cows SCA and SCDCA was 17.8 $\pm$ 6.9 μmol/l and 2.3 $\pm$ 1.0 μmol/l, respectively, while in milking cows SCA and SCDCA was 15.8 $\pm$ 5.7 and 2.3 $\pm$ 0.8 μmol/l, respectively. SCA concentration showed a characteristic change on the days immediately after calving: on calving day it was close to the mean SCA concentration found for dry cows, then it underwent a striking and abrupt rise and reached the value typical of post-parturient cows by post-partum (PP) day 4–5. Cholic acid was found to be the major primary bile acid in the blood of dairy cows. In dry cows the SCA : SCDCA ratio is 5:1. If the serum bile acid concentration rises, the SCA:SCDCA ratio will increase further.

Keywords: Bile acid, cholic acid, chenodeoxycholic acid, serum, dairy cow, reproductive status.

Bile acid determination has become known in laboratory diagnostics as a liver function test. It seems to be of interest to determine whether this method is suitable for diagnosing hepatic dysfunction also in dairy cows. To answer this question, one has to know the physiological characteristics of bile acid metabolism.

The blood serum levels of primary bile acids have so far been determined only in human beings. No data are available on serum primary bile acid concentrations of domestic animals.

On the other hand, several researchers have determined the total serum bile acid (SBA) concentrations in domestic animals and also in cattle.

Olsson (1986, 1988) monitored changes in the SBA concentrations of cows under physiological conditions. Gül and Gründer (1988) determined the SBA levels of cattle under physiological conditions and in numerous different diseases.

Materials and methods

Experimental design

Thirty cows were selected from a herd of Holstein-Friesian breed type and kept under loose housing conditions to determine the physiological levels

of serum cholic acid (SCA) and serum chenodeoxycholic acid (SCDCA). Average milk production per year was 6,800 kg. The cows were 4–6 years old and free from pathological changes as determined by physical and laboratory diagnostic examinations.

Blood samples were taken from the jugular vein on the spot, without disturbing the animals' natural biological rhythm, over a 24-h period. Blood

Table I

Feeding data of the experimental groups (per cow)

Experimental group	No. of animals	Dry matter, kg NE_1	Energy, MJ	Crude protein, kg	Crude fibre, kg
I	10	10.0	55	0.9	2.5
II	10	18.3	131	3.0	2.75
III	10	18.8	135	3.2	2.8

was taken from each cow at 4-h intervals on a total of 6 occasions during 24 h. The first sampling took place at 4:00 a.m.

The 30 cows were assigned to 3 groups of 10 cows each on the basis of their reproductive status. Group I included dry cows about 10–20 days before term. Group II consisted of cows that were 8–12 days after calving. Group III comprised high-yielding milking cows that were 10–15 weeks after calving. Their average daily milk production was 25–35 kg and the cumulative milk yield achieved in the previous lactation varied between 7,000 and 10,000 kg.

After the first study was ended, a fourth group (group IV) was formed from 10 down-calving cows of the same dairy farm. The aim of this second study was to determine changes in the SCA level in the peripartal period. Blood samples were taken from the cows from day 4 before to day 6 after calving daily, i.e. on 11 consecutive days, in the same hour of the day, and the SCA concentrations were determined.

The cows were fed 4 times at 4-h intervals during the daytime. The first feeding began at 4:00 a.m. and the last one at 4:00 p.m. Feeding data for group I–III cows are shown in Table I (data for groups I and II apply to cows of group IV).

Laboratory examinations

SCA and SCDCA concentrations were determined by radioimmunoassay (RIA). The test kits were obtained from the National "Frédéric Joliot-Curie" Research Institute of Radiobiology. The specificity of glyco-CA antiserum contained in the SCA test kit, expressed in % of cross-reaction, was as follows:

100% (glyco-CA), 117% (tauro-CA), 10% (CA), 5% (glyco-CDCA), 5% (tauro-CDCA), 1% (CDCA), 1% (glycodeoxycholic acid), and 0% (other steroids). The specificity of the glyco-CDCA antiserum contained in the SCDCA test kit was as follows: 100% (glyco-CDCA), 90 % (tauro-CDCA), 20% (CDCA), 0.4% (glyco-CA), 0.5% (tauro-CA) and 0% (other steroids). Thus, the test kits are suitable for combined measurement of the glyco- and tauro-conjugates of CA and CDCA. The lowest measurable bile acid concentration was 0.15 μmol/l with both test kits (Orbán, 1984; Ungár et al., 1986).

Ketone body concentration of the urine was determined by Ross' method. Serum aspartate aminotransferase (AST) activity and total cholesterol (TCh) level were determined by colorimetry, with the test collection of REANAL Fine Chemicals Co. (Budapest). Blood glucose was measured by Trinder's (1969) method, and free fatty acid concentration was determined by the colorimetric method described by Duncombe (1964).

Statistical evaluation of the data was done by two-sample *t* test and correlation analysis.

Results

Mean SCA and SCDCA concentration was 7.8 ± 3.3 and 1.5 ± 0.7 μmol/l, respectively, in group I (Fig. 1). The mean total concentration of the two primary bile acids was 9.3 ± 2.0 μmol/l and the SCA : SCDCA ratio was 5.1 : 1.

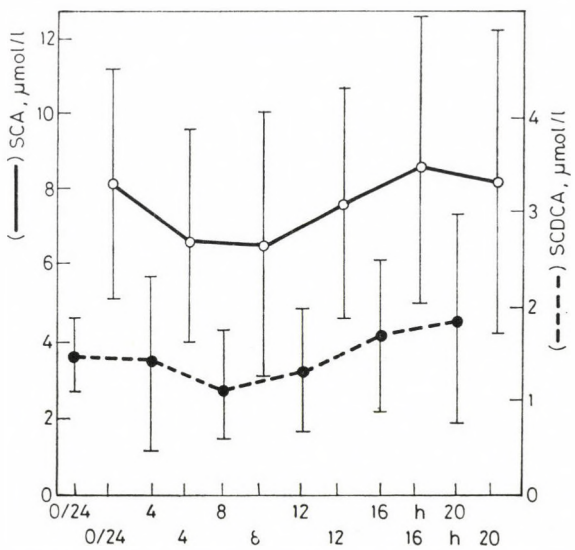

Fig. 1. Serum cholic acid (SCA) and serum chenodeoxycholic acid (SCDCA) levels in dry cows (group I). Solid line: SCA (μmol/l); broken line: SCDCA (μmol/l)

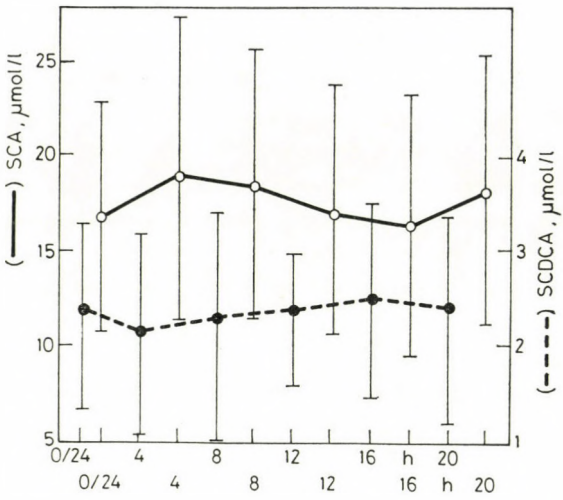

Fig. 2. Serum cholic acid (SCA) and serum chenodeoxycholic acid (SCDCA) levels in freshened cows (group II). Solid line: SCA (μmol/l); broken line: SCDCA (μmol/l)

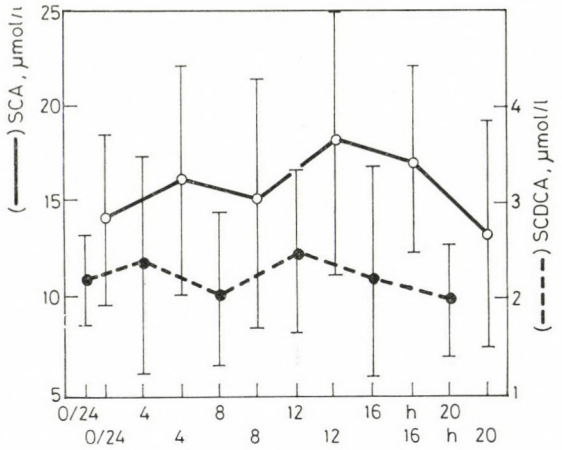

Fig. 3. Serum cholic acid (SCA) and serum chenodeoxycholic acid (SCDCA) levels in milking cows at the peak of lactation (group III). Solid line:SCA (μmol/l); broken line: SCDCA (μmol/l)

In group II mean SCA and SCDCA concentration was 17.8 ± 6.9 and 2.3 ± 1.0 μmol/l, respectively (Fig. 2). The mean total concentration of the two primary bile acids was 20.1 ± 3.9 μmol/l and the SCA : SCDCA ratio was 7.9 : 1.

In group III mean SCA and SCDCA concentration was 15.8 ± 5.7 and 2.3 ± 0.8 μmol/l, respectively (Fig. 3). The mean total concentration of the two primary bile acids was 18.1 ± 3.2 μmol/l and the SCA : SCDCA ratio was 7 : 1.

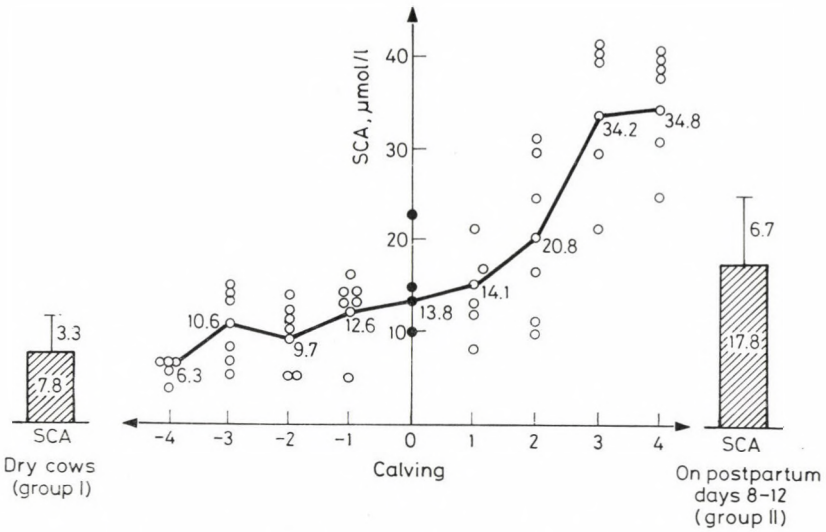

Fig. 4. Changes in SCA level in the peripartal period (group IV)

In group IV mean SCA concentration was 4.75 ± 2.0, 7.4 ± 3.45 and 16.6 ± 5.8 μmol/l 4 days before calving, on the day of calving and 5 days after calving, respectively (Fig. 4).

AST activity, cholesterol, blood glucose and FFA levels were in the physiological range. Among the postparturient cows there was no ketonuric animal.

Discussion

The changes found in the serum levels of primary bile acids in healthy dairy cows can be characterized as follows.

1. *SCA and SCDCA levels are high.* In dry cows (group I) the mean SCA and SCDCA concentration was found to be 7.8 ± 3.3 and 1.5 ± 0.7 μmol/l, respectively. In human beings SCA and SCDCA concentration is as low as 0.46 ± 0.26 and 0.63 ± 0.32 μmol/l, respectively (Ungár et al., 1986). The SCA concentration is 2.2 times as high as that of human beings.

This finding is consistent with the observation that ruminants have strikingly higher total serum bile acid (SBA) levels than do e.g. carnivores. The SBA level of fasting dogs was found to be 2.3 ± 0.4 μmol/l, and after feeding SBA level rose to 8.3 ± 2.2 μmol/l (Center et al., 1984). In contrast, the SBA level of dairy cows was found to be 37.4 ± 25.5 μmol/l by Olsson (1988) and 22.3 ± 11.0 μmol/l by Gül and Gründer (1988). Sheep also have high SBA levels: West et al. (1987) reported an SBA level of 25.0 ± 2.1 μmol/l.

The uniquely high serum bile acid level of ruminants is all the more

remarkable as their feed contains little fat and, thus, in ruminants bile acids have a less important role in intestinal fat digestion than they do in carnivores.

2. *SCA level rises immediately post partum.* In cows whose SCA level was monitored daily in the peripartal period (group IV), a substantial rise of SCA concentration was demonstrated after calving (Fig. 4). On the day of calving, and even on postpartum day 1, SCA concentration was the same as the mean concentration measured in dry cows. SCA concentration started to rise on postpartum day 2, reaching the mean value typical of postparturient cows by postpartum day 4–5.

The elevation of the bile acid level of the blood serum may be of hepatic origin and associated with fatty degeneration of the liver characteristic of high-yielding dairy cows at that time. This phenomenon fits into the tendency outlined by other laboratory parameters indicating the liver function and status of postparturient cows. Namely, it is known that in the blood of postparturient cows the activities of hepatocellular enzymes are higher, the cholesterol level is lower, and prothrombin time as well as Bromsulphalein retention time are longer than in dry cows.

3. *Milking cows have higher SCA and SCDCA levels than dry cows.* In the group of freshened cows (group II) mean SCA concentration was 17.8 ± 6.7 μmol/l and mean SCDCA concentration was 2.3 ± 1.0 μmol/l, i.e. 22 and 1.5 times higher than the corresponding values of dry cows.

In milking cows (group III) mean SCA and SCDCA concentration was 15.8 ± 5.7 and 2.27 ± 0.8 μmol/l, respectively, i.e. similar to that measured for freshened cows and higher than that measured for dry cows. A slight decrease of serum cholic acid level was noted.

When determining the physiological SBA level, also other researchers reported substantial differences between groups of animals even within the same species. Gül and Gründer (1988) found 40% lower SBA level in calves than in dairy cows. According to Olsson (1988), SBA level is the highest in lactating cows and declines towards the end of lactation. The cause of this phenomenon is not known satisfactorily yet. The substantial rise of SBA, SCA and SCDCA in lactating cows may be attributed to factors associated with high milk production and exerting a lasting effect on bile acid metabolism. We can agree with Olsson (1988) that after calving the quantitative and qualitative changes of the feed exert an effect on the absorption and enterohepatic circulation of bile acids, resulting in their elevated concentrations in the blood serum.

4. *The mean SCA and SCDCA levels are characterized by substantial standard deviation.* In groups I–III this standard deviation was between 38.6 and 46.4%.

It should be mentioned that the mean SCA and SCDCA levels of human beings also show high standard deviation (72 and 51%, respectively).

Gül and Gründer (1988) reported 49% while Olsson (1988) 68% standard deviation for the mean SBA level of dairy cows. Owing to the high standard deviation and very distant extreme values, Olsson (1988) doubts that bile acid determination could be used for diagnostic purposes in dairy cows as reliably as it can in human beings and monogastric animals. It is worthy of mention, however, that Johnson et al. (1985), who reported an SBA level of 1.8 ± 1.3 μmol/l for fasting dogs, found a standard deviation as high as 72%.

It should be pointed out that both Gül and Gründer (1988) and Olsson (1988) gave the mean values for dairy cows in general and did not differentiate between dry, freshened and milking cows. By such differentiation the standard deviation and the difference between the extreme values could surely be diminished.

5. *The diurnal variation of the SCA and SCDCA levels is little and not influenced by the time of feeding.* Deviations larger than 12% from the mean SCA and SCDCA level were found in none of the groups, and no appreciable change was demonstrated in the serum level of primary bile acids after feeding.

According to Olsson (1988), the SBA level shows no substantial diurnal variation and it does not change appreciably even after a 24-h fast. He observed no influence of the feeding time on diurnal variations of the SBA level. SBA level was not found to depend on the season; neither were there big differences between cow herds.

As opposed to dairy cows, in carnivores the time of feeding markedly affects the serum level of bile acids. Bile acid level is the lowest in fasting dogs, then it undergoes a twofold increase by 2 h after feeding.

The difference found between ruminants and carnivores in the influence of feeding on bile acid level is not surprising as in ruminants the abomasal contents are continuously passed on into the small intestine. Thus, periodical small intestinal digestion and the abrupt influx of large volumes of bile into the intestine need not be reckoned with in ruminant animals.

6. *The ratio of SCA and SCDCA levels in cows differs from that reported for human beings.* In dry cows the SCA : SCDCA ratio is 5 : 1, while in human beings it is 0.8 : 1. This indicates that in cows cholic acid is the major primary bile acid and chenodeoxycholic acid is present only in rather small quantities.

If serum bile acid concentration rises in cows, SCA will undergo a greater increase than SCDCA. This can be observed in freshened cows in which the SCA : SCDCA ratio is already 9 : 1. In cows with pathologically elevated bile acid level this ratio may reach 13–15 : 1.

For human beings the reverse is true: the elevation of SBA is accompanied by a shift in the ratio of the two primary bile acids in favour of chenodeoxycholic acid.

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CHARACTERIZATION OF *ACTINOBACILLUS* STRAINS ISOLATED FROM ABORTED SWINE FETUSES

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Eleven *Actinobacillus* strains were isolated in pure culture from 12- to 13-week-old aborted swine fetuses. Apart from minor biochemical differences, they resembled strains isolated from sow vaginas by Ross et al. (1972) in the U. S. A. Some of the strains agglutinated sheep, cattle and horse but not pig, dog and chicken erythrocytes. Haemagglutination was mannose resistant and could be inhibited by specific hyperimmune serum. Heating above 70 °C diminished or abolished haemagglutination of the cultures. Electron microscopy showed no fimbriae on the surface of the bacteria.

Keywords: Actinobacillus, sow, abortion

Members of the genus *Actinobacillus* cause sporadic diseases involving suppuration and septicaemia in farm animals (Biberstein, 1981). This genus includes *A. lignieresii*, *A. equuli*, *A. suis*, *A. capsulatus* and *A. actinomycetemcomitans* (Biberstein, 1981; Mannheim et al., 1984). In addition, it has been suggested that strains isolated from sporadic, haemorrhagic, necrotic pneumonia of swine which resemble *Pasteurella haemolytica*, together with the species *Haemophilus pleuroneumoniae*, should be reclassified within the genus *Actinobacillus* as two biotypes of a newly created species, *A. pleuropneumoniae* (Pohl et al., 1983). Mráz (1969) suggested to place *P. haemolytica* into this genus as well, under the name of *A. haemolyticus*. In the U. S. A., Ross et al. (1972) isolated from the vagina of sows strains which they regarded as a new species of *Actinobacillus* after comparison with other members of the genus. In some aspects similar strains were isolated by Mair from swine fetuses and genitals and named *Pasteurella* sp. (cit. Mannheim et al., 1980; Sneath and Stevens, 1985).

This paper reports on the characterization of the cultural, biochemical and serological features of *Actinobacillus* strains isolated from aborted swine fetuses in Hungary.

Materials and methods

Bacteria

Bacterial strains were isolated from the parenchymal organs and stomach contents of swine fetuses aborted at the 12th–13th week of gestation. The samples were inoculated onto nutrient agar and sheep blood agar containing 0.5% yeast extract. Agar plates were incubated at 37 °C for 24 h. Stomach contents were inoculated onto two blood agar plates, one of which was incubated for 24 h in air, the other for 7 days in air with 10% CO_2 content. Smears of stomach contents were stained by Gram's and Stamp's methods.

One *Actinobacillus* sp. strain of Ross (A56) and two *Pasteurella* sp. strains (All and A204) received from Dr. P. H. A. Sneath, Department of Microbiology, Leicester University, Leicester, U. K. (Sneath and Stevens, 1985) were included in the examinations.

Samples from liver and kidney were stained by Levaditi's silver-impregnation method to detect leptospirae.

Biochemical tests

Biochemical examination of the isolates was carried out according to Biberstein (1978).

Haemagglutination

Haemagglutinating ability of the strains was tested on slides and microtitre plates using 1% suspensions of washed sheep, pig, dog, horse, cattle and chicken erythrocytes. One drop of saline was mixed on a slide with one colony from a blood agar plate, then an equal volume of red blood cell suspension was added. In a positive reaction the erythrocytes clumped within a few seconds. When microtitre plates were used, equal volumes (50 μl) of doubling dilutions of bacterial suspension (MacFarland 2) washed off a blood agar plate or broth culture and erythrocyte suspension were dropped into each well. The test was read after 3 h at room temperature. Resistance of the haemagglutinin to 1% mannose was also tested.

Heat susceptibility of the haemagglutination was examined by heating the broth cultures in a waterbath at 50, 60, 70, 80 and 90 °C for 30 min or boiling for 10 min, then testing for haemagglutinating ability as previously.

Production of antiserum

Hyperimmune serum was produced against one of the haemagglutinating strains by intravenous injection of a rabbit at 3- to 4-day intervals with 0.5, 1, 2, 3, 3 and 3 ml broth culture inactivated with 0.15% formalin, followed by exsanguination of the rabbit 7 days after the last injection.

Haemagglutination inhibition test

The haemagglutination inhibition test was carried out on microtitration plates. To doubling dilutions of the hyperimmune rabbit serum the same volume (50 μl) of broth culture was added. The plates were incubated at 37 °C for 45 min, then 100 μl of 1% erythrocyte suspension was added to each well. The test was read after 3 h at room temperature.

Electron microscopy

One drop of a slightly opalescent broth culture of the bacterium was mixed with 1% w/v aqueous solution of phosphotungstic acid on a Formwar coated copper microgrid and examined with a Phillips 201 CS type transmission electron microscope.

Results

Bacteria of similar appearance were isolated in pure culture from 11 fetuses of 4 sows in different herds. Growth was obtained with incubation both after 24 h in air and 7 days in air containing 10% CO_2. Colonies on blood agar at 24 h were grey, convex, smooth and approximately 1–2 mm in diameter; they did not stick to the surface of the agar. A narrow β-haemolytic zone was present around the colonies. Microscopically, the organisms appeared as Gram-negative rods of 1–2 μm length. Growth on nutrient agar was poor. Stomach content smears and histological examination of livers and kidneys revealed no other organisms.

With the exception of sucrose fermentation, ornithine decarboxylase activity and growth on MacConkey agar, the strains were biochemically uniform (Table I).

Six strains, isolated from 2 litters, agglutinated sheep, cattle and horse but not pig, dog and chicken erythrocytes: the remaining 5 strains (isolated from other 2 litters) failed to agglutinate any erythrocyte. The haemagglutinating titre of broth cultures reached 1 : 64. The haemagglutination was not inhibited by 1% mannose.

The rabbit immune serum inhibited the haemagglutination of all strains at a titre of 1 : 1280.

Haemagglutinating activity could be reduced or abolished by heating. Treatment at 50 °C and 60 °C for 30 min had no effect but heating at 70 °C or 80 °C for 30 min reduced the haemagglutination titre by one dilution to 1 : 32. Exposure to 90 °C for 30 min or boiling for 10 min abolished haemagglutinating activity of the strains completely.

By electron microscopic examination fimbriae could not be detected on the surface of the bacteria (Fig. 1). The haemagglutinating bacteria adhered closely to the surface of the red blood cells.

Table I

Cultural and biochemical features of 11 *Actinobacillus* strains isolated from aborted swine fetuses, *Actinobacillus* sp. A56 and *Pasteurella* sp. A11 and A204

Feature	Own isolates 11 strains	A56	A11 and A204
Gram staining	−	−	−
Motility	−	−	−
Catalase	+	+	+
Oxidase	+	+	+
OF test	fermentative	fermentative	fermentative
Haemolysis	β	β	β
Growth on MacConkey agar	− (+2)	+	−
Indole	−	−	−
Urease	+	+	+
H_2S (Pb acetate strip)	+	+	+
Nitrate reduction	+	+	+
ONPG	+	+	+
Ornithine decarboxylase	d (+6)	−	+
Lysine decarboxylase	−	−	−
Arginine dihydrolase	−	−	−
Fermentation of			
arabinose	+	+	+
glucose	+	+	+
lactose	+	+	+
mannitol	+	+	+
raffinose	−	−	−
salicin	−	−	−
sucrose	d (+6)	−	+
trehalose	−	−	−
xylose	+	+	+

Discussion

The exact identification of strains within the genera *Actinobacillus*, *Haemophilus* and *Pasteurella* is very difficult because of their close relationship and common features (Mannheim et al., 1980; Mannheim et al., 1984; Sneath and Stevens, 1985).

Based on their morphological, cultural and biochemical characteristics the 11 strains isolated by us from aborted swine fetuses seem to belong to the genus *Actinobacillus* (Mráz, 1983; Sneath and Stevens, 1985). These strains differed from *A. lignieresii* and *A. equuli* in haemolytic activity, catalase positivity, and production of H_2S. Furthermore, they did not adhere to the agar and did not ferment trehalose and raffinose (Mannheim et al., 1980; Mann-

heim et al., 1984; Sneath and Stevens, 1985). In comparison to *A. suis* they formed smaller colonies and fermented mannitol but not raffinose, salicin or trehalose (Dorssen and Jaartsveld, 1962; Zimmermann, 1964). Based on haemolysis on sheep blood agar, H_2S production, lactose, raffinose, salicin and trehalose fermentation they differed from *A. capsulatus* and *A. actinomycetemcomitans* (Mannheim et al., 1980; Mannheim et al., 1984; Sneeth and Stevens, 1985). Their strong urease activity was suggestive of Taxon 12 of Bisgaard and *P. ureae*. The latter has been isolated previously from aborted swine fetuses (Corkish and Naylor, 1982), but our strains were less fastidious, grew better on blood agar, fermented lactose and xylose, and produced H_2S (Mannheim et al., 1980; Mannheim et al., 1984; Sneath and Stevens, 1985). In contrast to Bisgaard's Taxon 12 arabinose and xylose but not raffinose, salicin and trehalose were fermented (Bisgaard, 1986). The strains differed from *P. haemolytica* biotype A in producing urease and H_2S and in fermenting raffinose (Biberstein, 1978). Catalase positivity differentiated them from the *P. haemolytica*-like strains isolated from pigs and recently identified as *A. pleuropneumoniae* biotype 2 (Bertschinger and Seifert, 1978; Pohl et al., 1983). Our strains are excluded from Bisgaard's Taxon 15 on the basis of urease activity (Bisgaard, 1984). Only slight differences were apparent between our strains and those isolated by Ross et al. (1972) from the vagina of sows, therefore they seem to belong to the same species. Six isolates fermented sucrose and produced ornithine decarboxylase unlike *Actinobacillus* sp. described by Ross (1972) and in this aspect showed the same characteristics as *Pasteurella* sp. of Mair (cit. Sneath and Stevens, 1985). These two species show close relationship (Sneath and Stevens, 1985) and in our view they might be regarded as two biotypes of one species; however, DNA-DNA hybridisation tests have not been carried out yet to prove it.

Six out of the 11 strains agglutinated sheep, horse and cattle but not pig, dog and chicken erythrocytes. There was no correlation between the haemagglutination and any biochemical reactions. Haemagglutinating strains could be detected both among sucrose and ornithine decarboxylase negative and positive strains, suggesting a close relationship between *Pasteurella* sp. of Mair (cit. Sneath and Stevens, 1985) and *Actinobacillus* sp. of Ross (Ross et al., 1972).

The haemagglutinin was heat labile, and a specific immune serum of high titre inhibited its activity. Fimbriae have been revealed neither on the surface of haemagglutinating nor on that of non-haemagglutinating strains. In the case of haemagglutinating strains a thermolabile surface antigen proved to be responsible for the haemagglutination, as it occurs in other species (Varga et al., 1987). There is no evidence whether these adhesins have any role in the pathogenicity of these strains.

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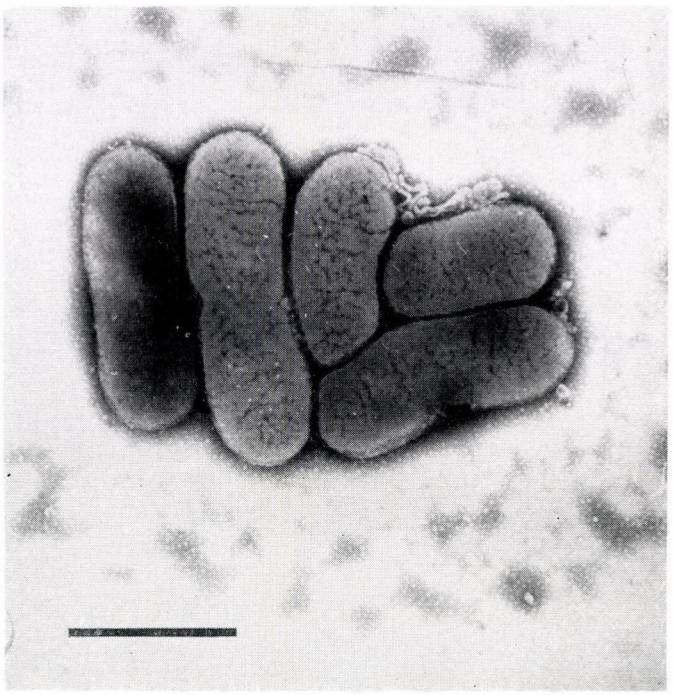

Fig. 1. Bacterial cells without fimbriae adhere to each other (bar = 10 μm)

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SURVEY OF *ACTINOBACILLUS (HAEMOPHILUS) PLEUROPNEUMONIAE* INFECTION IN SWINE BY DIFFERENT METHODS

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Lung and serum samples from pigs that died or were emergency-slaughtered in a pooled, conventional fattening herd were examined to survey *Actinobacillus pleuropneumoniae* infection and to compare the sensitivity of different testing methods. A total of 110 lungs were used for cultural isolation of the agent and direct immunofluorescence (IF) of impression smears. Boiled lung suspensions were tested by coagglutination (Co-A) and agar gel precipitation (AGP). Eighty-seven sera were tested along with lung samples from the same pigs.

The lungs yielded a varied bacterial flora most often containing *Pasteurella multocida* and less frequently *Actinomyces (Corynebacterium) pyogenes*, *E. coli* and *Salmonella*. *A. pleuropneumoniae* was isolated from 30 lungs: from 22 lungs it grew out in pure culture, from 7 as mixed culture with *P. multocida* and from 1 as mixed culture with *A. pyogenes*.

The number of positive samples obtained by the different methods was as follows: coagglutination test (with boiled lung suspensions): 63 (57.3%); immunofluorescence: 43 (39.2%); AGP test (with serum): 31 (35.6%); AFP test (with boiled lung suspension): 25 (22.7%).

A total of 23 samples (20.7%) were negative by all serological tests and by cultural isolation. Most samples gave positive results by two or more tests while 26 samples only by one test (most often, on 13 occasions, by the Co-A test).

The Co-A test detected antigenic components of serotypes that have not been isolated in Hungary so far. This indicates that it is not enough to test one strain from a given lung sample: several colonies must be cultured and serotyped.

In the herd surveyed serotype 2 was dominant but serotypes 1, 3, 5, 7, 8, 10, 11 and 12 also occurred.

The Co-A test proved to be the most sensitive of the five methods compared. Other methods, however, can serve as useful complementary tests.

Keywords: Actinobacillus, Haemophilus, *A. pleuropneumoniae*, pig, pleuropneumonia, comparative study, coagglutination test, agar gel precipitation, cultural isolation, direct immunofluorescence

Pleuropneumonia of swine caused by *Haemophilus pleuropneumoniae* was first described in 1964 (Shope et al., 1964). Based on its DNA structure, the causative agent has been transferred to the genus *Actinobacillus* and is referred to as *Actinobacillus pleuropneumoniae* (Pohl et al., 1983).

So far 12 different serotypes of *A. pleuropneumoniae* have been reported (Nielsen, 1986). Some serotypes are antigenically closely related (Gunnarson et al., 1978; Mittal et al., 1988*a*, 1988*b*). At the same time, within certain serotypes subtypes may occur (Nielsen, 1986). The degree of antigenic related-

ness between serotypes varies: a close relationship was found among serotypes 1, 9 and 12, between serotypes 4 and 7 and among serotypes 3, 6 and 8 (Nicolet, 1988).

Numerous tests have been used for serological identification of *A. pleuropneumoniae* infection and for its serotype determination. Earlier the complement fixation test was used most widely (Nicolet et al., 1971; Nielsen, 1974; Gunnarson, 1979). The latex agglutination test (Mitui et al., 1981), ELISA (Nicolet et al., 1981), counterimmunoelectrophoresis (Piffer et al., 1986) and direct immunofluorescence were used by relatively few authors though were reported to have high sensitivity and specificity. Many authors (Mittal et al., 1983*c*; Nicolet et al., 1981; Nielsen and O'Connor, 1984; Nicolet, 1988) found that the indirect haemagglutination test was specific and sensitive.

The tube agglutination and agar gel precipitation (AGP) tests have been compared by many of the above-mentioned authors. The general view is that in the agglutination test cross-reactions between different serotypes are very common, while AGP, though less sensitive, is one of the most specific tests.

The coagglutination (Co-A) test has been used for identification of *A. pleuropneumoniae* infection and for serotype determination with very good results (Mittal et al., 1983*a*, 1983*b*; Mittal et al., 1987; Hunter and Livingstone, 1986; Skollova and Gois, 1987).

The purpose of the work reported here was to collect data on the value of Co-A, to compare its sensitivity with that of other tests and to use it for surveying *A. pleuropneumoniae* serotypes occurring in Hungary.

Materials and methods

Test material

The test material was collected at a pig farm where 30,000 to 50,000 pigs are fattened at a time. These pigs are purchased from large pig farms all over Hungary at a body mass of 25–30 kg. The test material was collected at the farm's slaughterhouse from pigs slaughtered for some reason (87 culled or emergency-slaughtered pigs; lung and blood samples) and from pigs that had died of pneumonia (23 pigs; only lung samples). The 110 lung samples were used for bacterial culture, the impression smears made from them were tested by IF using a conjugate against serotype 2, and boiled lung suspensions were tested by Co-A against the 12 known serotypes and by AGP against serotypes 1 and 2. The 87 sera were tested by AGP.

Bacterial culture

The samples were inoculated onto blood agar plates with a streak of *Staphylococcus aureus*. Suspect colonies were inoculated onto tryptic soy agar

(TSA) containing 5% yeast extract. After obtaining a pure culture, the strain was again inoculated onto blood agar with a streak of *S. aureus*.

The isolate was considered *A. pleuropneumoniae* if it required the presence of *S. aureus* colonies for its growth and was a Gram-negative, non-ciliated, haemolytic coccobacillus. Biochemical tests were done on only few strains.

IF test

Impression smears made from the lung sections were examined by direct IF as usual. The conjugate was prepared by us as follow. Rabbits were immunized with the whole-cell suspension of a serotype 2 strain isolated in Hungary. Immune sera of 1 : 2048–1 : 4096 agglutination titre were obtained and used for conjugate preparation. The optimal dilution of the conjugate was 1 : 32.

Coagglutination test

Pieces from the lungs were stored at —20 °C until used. After thawing, about 2 g pieces were homogenized in 3 ml saline, the suspension was filled into a Wassermann tube and boiled in water-bath for 10 min. Subsequently the suspension was centrifuged at 8000 *g* for 30 min and the supernatant was used for the Co-A and AGP tests.

The details of the preparation of Co-A reagents and the procedure of the Co-A test have been described earlier by Mittal et al. (1983*a*, 1983*b*).

Agar gel precipitation (AGP) test

Eighty-seven blood sera and 87 lung extracts were examined by AGP test carried out in the usual way. Veronal buffer (pH 8.6) containing 1% agarose was layered on a slide. The distance between the wells was 6 mm and the diameter of the wells was 4 mm. The slides were incubated in moist chamber at room temperature and the test was read after 24, 48 and 72 h.

Results

Only 4 out of the 110 lung samples examined were free from gross pathological lesions. The lesions varied widely in severity, extent and type. Necropsy findings typical of *A. pleuropneumoniae* (acute or chronic haemorrhagic pneumonia, necrotic foci, acute fibrinous or chronic fibrous pleuritis) were seen in 61 lungs, mainly in the upper third of the diaphragmatic lobe, near the obtuse margin.

A. pleuropneumoniae was isolated from 30 out of the 110 lung samples examined. The 30 lung samples yielding *A. pleuropneumoniae* included 4 lungs in which no lesions indicative of this pathogen were seen. *A. pleuropneumoniae*

grew out in pure culture from 22 lung samples and in mixed culture with *P. multocida*, *Actinomyces (Corynebacterium) pyogenes* and *E. coli* from 8 lung samples. Only 22 lungs failed to yield bacteria by cultural isolation; from the other lung samples the above-mentioned bacteria (most frequently *P. multocida*) and, in one case, a closely not identified *Salmonella* species grew out. The serotype distribution of the 30 *A. pleuropneumoniae* isolates as determined by AGP was as follows: 26 strains belonged to serotype 2, 2 strains were mixed serotypes (2 and 11), while 2 strains could not be typed.

On the basis of the Co-A test of boiled lung suspensions, the distribution of serotypes in the 63 positive samples was as follows: only serotype 1: 2 strains; only serotype 2: 23 strains; only serotype 5: 1 strain; only serotype 8: 4 strains; only serotype 10: 3 strains; only serotype 11: 3 strains; mixed (more than one serotype): 27 strains.

The prevalence of different serotype components in the 27 samples which contained the antigenic components of 2 or 3 serotypes was as follows: serotype 1 was present in 9 samples, serotype 2 in 19, serotype 3 in 8, serotype 5 in 8, serotype 7 in 1, serotype 8 in 3, serotype 10 in 1, serotype 11 in 11, and serotype 12 in 1 samples.

AGP test of boiled lung suspension was done only with immune sera produced against serotypes 1 and 2. The 25 samples positive in this test showed the following distribution: in 9 samples only serotype 1, in 12 samples only

Table I

Comparison of different tests for sensitivity of detecting *A. pleuropneumoniae* infection

Method	Number of samples	Positive, total	Positive in both tests	Positive in first test, negative in second test[1]	Negative in first test, positive in second test
Co-A[2]–IF[3]	110	75	31	32	12
Co-A–AGP[4] (with serum)	87[6]	59	21	28	10
Co-A–cultural isolation[5]	110	73	20	43	10
Co-A–AGP (with boiled lung suspension)	110	72	16	47	9
IF–AGP (with serum)	87	51	13	20	18
IF–cultural isolation	110	47	26	17	4
IF–AGP (with b.l.s.[7])	110	57	11	32	14
AGP (serum) – cultural isol.	87	41	10	21	10
AGP (serum – AGP (b.l.s.)	87	41	5	26	10
cultural isolation – AGP (b.l.s.)	110	48	7	23	18

[1] e.g. (first line): Co-A positive, IF negative; [2] Co-A = coagglutination test with boiled lung suspension; [3] IF = direct immunofluorescence test; [4] AGP = agar gel precipitation test; [5] successful isolation; [6] as only 87 sera were available for testing, only the 87 lung samples taken from pigs whose serum samples were available were used; b.l.s. [7] boiled lung suspension

serotype 2 antigenic components were detected, while 4 samples contained mixed (serotype 1 and 2) antigenic components.

Comparison of the different serological tests for sensitivity is presented in Table I. The Co-A test proved to be the most sensitive. This finding is supported, from different aspects, by data shown in Tables II, III and IV.

Table II

Number and ratio of samples positive in the different tests

Method	Number of samples, total	Positive samples	
		n	%
Co-A	110	63	57.3
IF	110	43	39.2
AGP (with serum)	87	31	35.6
Cultural isolation	110	30	27.3
AGP (with boiled lung suspension)	110	25	22.7

Table III

Distribution of results negative by all tests and positive by a single test

	n	%
Negative by all five tests	23	20.7
Positive only by cultural isolation	1	0.9
Positive only by IF	2	1.8
Positive only by AGP with boiled lung suspensions	3	2.7
Positive only by AGP with blood serum	7	6.3
Positive only by Co-A	13	11.8

Table IV

Performance of the different tests (if pigs positive by at least one test are considered positive)

Test	Performance, %
Co-A	72.45
IF	49.45
AGP (with serum)	46.81
Cultural isolation	34.50
AGP (with boiled lung suspension)	28.75

Discussion

Before this study, no Hungarian data were available on the performance of the coagglutination test and the international literature also contained only few reports. This fact prompted us to collect data on the sensitivity and diagnostic applicability of the test.

The pigs kept at the fattening farm surveyed by us had been derived from different areas of Hungary, from numerous different pig herds. It was, therefore, not surprising that their lungs yielded a varied bacterial flora by cultural isolation. The pigs were at different stages of *A. pleuropneumoniae* infection. This is easy to explain, as some pig groups had obviously been infected already when transferred to the fattening farm, while others had come from infection-free herds. The length of the period the pigs spent at the fattening farm up to death or emergency slaughter also differed, as evidenced by the body mass of the examined pigs which varied between 30 and 100 kg.

In several cases *A. pleuropneumoniae* could not be isolated despite gross pathological lesions indicative of its presence. Several explanations exist for this phenomenon: the bacteria had been eliminated from the lungs (perhaps by medication); the lung portion used for isolation did not contain the bacterium though the agent was present in other parts of the lungs; or the growth of *A. pleuropneumoniae* was suppressed by some other, more quickly growing bacterium. In some instances *A. pleuropneumoniae* was isolated from unchanged or catarrhal areas of the lungs. This may have been due to fresh infection or to the fact that the pig had acquired protection as a result of an earlier mild infection (or, possibly, vaccination) but became a permanent carrier of the bacterium.

From positivity rates obtained by the different methods used (Tables I and III) it can be seen that no method gave results fully identical with those of any other method used. Some results are easy to explain while others are difficult to interpret. When only the AGP test of serum samples was positive, this must have been due to the fact that the bacteria had been eliminated from the organism. The number of samples positive exclusively by IF, a procedure considered sensitive, was only 2 (Table III), possibly because only a conjugate prepared from serotype 2 was used. A rather surprising finding is that while AGP of boiled lung suspensions gave the lowest positivity rate (25 positive samples, 22.7% positivity rate), in 3 cases it was the only test that detected infection.

The results clearly show that the Co-A test is highly sensitive: it proved the most sensitive method in all comparisons (Tables I–IV). Besides, being simple, rapid and inexpensive, it is highly suitable for use in the diagnostic practice. Preparation of the staphylococcal extract is undoubtedly a laboursome process. At the same time, it is a great advantage that this reagent can be

prepared in large quantities at a time, as at 4 °C it can be stored for months without any loss of its efficacy. It is important to mention that extracts from autolysed samples or those stored in formalin can also be used in this test. The safety of detection can be increased by complementing the Co-A test with other methods, as sometimes precisely a less sensitive method detects infection.

Earlier only *A. pleuropneumoniae* strains belonging to serotypes 1 and 2 were isolated and the vaccine available in Hungary contains only these two serotypes. Some strains belonging to serotypes 3 and 9 have been isolated only recently. Although serotype 2 was found to be the dominant serotype also in this study, the antigenic components of all serotypes except serotypes 4, 6 and 9 were detected (though serotypes 7, 10 and 12 only once). The simultaneous occurrence of different serotypes in pig herds collected from different farms is not surprising but such highly varied occurrence as seen in this case was unexpected. Only serotypes 1 and 2 have been isolated. This question should be clarified by further studies.

The growth rate of the different serotypes and, moreover, different strains within the same serotype was found to differ considerably. Strains of serotype 2 grow most quickly: thus, in the cultures (and perhaps also in the pig lungs) they suppress the growth of other serotypes. On the other hand, several authors reported that by extending the surveys more and more new serotypes were isolated. In Japan, for a long time only serotype 2 was isolated. Subsequently serotype 5 became more and more common, and recently serotypes 1, 6 and 7 have also been reported to occur (Kume and Nakai, 1988). In Austria, Baumgartner et al. (1989) tested the sera of slaughtered sows by complement fixation using antigens of serotypes 1–10 and demonstrated the presence of antibodies to all serotypes. Although the results presented here do not permit an exact determination of the role played by antigenic relationship among serotypes, they indicate that the occurrence of new serotypes must be reckoned with.

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OCCURRENCE OF MONOGENEANS ON FRESHWATER FISHES IN IRAN: *DACTYLOGYRUS* SPP. ON CULTURED IRANIAN FISHES

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Thirteen *Dactylogyrus* spp. were found on cultured fishes in five Iranian fish farms. Besides typical *Dactylogyrus vastator*, specimens with small anchors were often found in goldfish, which were designated as *D. vastator forma minor. Rutilus frisii kutum*, a fish cultured only in Iran, was infected by two *Dactylogyrus* spp. during its short period of prerearing in Iranian fish farms. All *Dactylogyrus* found are new for the Iranian fauna.

Keywords: Monogenea, *Dactylogyrus* spp., freshwater fishes, Iran

Only few data are available on parasitic infections and diseases of Iranian cultured fishes. Mokhayer (1976 and 1985) reported on some pathogenic fish parasites. No information exists about monogeneans.

Of the neighbouring regions, in the Soviet Union the parasitic infections of cultured fishes are well studied. The parasite fauna of fish was reviewed by Agapova (1966) in Khazakhstan and by Mikhailov (1975) in Azerbaidzhan. A comprehensive work on diseases and parasites of cultured fishes was written by Bauer et al. (1981). A work by Salih et al. (1988) on the helminth fauna of pond fishes of Iraq may concern similar problems as those existing the neighbouring, south-western region of Iran.

The occurrence of *Dactylogyrus* spp. on the gills of Iranian fishes is reported in this paper.

Materials and methods

The common carp *(Cyprinus carpio)* is the main fish of Iranian carp farms, but introduced "Chinese or herbivorous" fishes (grasscarp, *Ctenopharyngodon idella;* bighead, *Aristichthys nobilis;* silver carp, *Hypophthalmichthys molitrix*) are often raised in polyculture. Herbivorous fishes were first imported from the Soviet Union and placed into the Anzali lagoon (Caspian water system) in 1971. Later on, in 1982 3–4 million larvae of common carp and Chinese carps were brought in from Romania and in 1987 about 800 breeders of the

same fish species were imported from Hungary. Besides common carp and Chinese carps, a good-quality fish *(Rutilus frisii kutum)* is readily cultured in some Iranian pond farms: specimens of this fish are hatched and reared for four months before released into rivers flowing into the Caspian Sea. The gibel carp *(Carassius auratus gibelio)* as a wild fish very often inhabits the ponds and irrigating channels of fish farms. In addition to table fishes, the goldfish *(Carassius auratus)* is also cultured in some Tehran fish farms which are supplied by underground water.

Samples of fish were collected in 5 fish farms (Sangar and Sameskandeh in the Caspian water system; Esfahan, which drains its water into sodium lakes in Central Iran; farms in Khozestan belonging to the Gulf region; and a farm close to Tehran).

Fish were examined in different seasons of the year, and specimens of different size were studied. Only positive cases (*Dactylogyrus*-infected fish) were recorded.

Fish were examined either at the fish farm or taken to a laboratory alive for a more thorough examination. Parasites were collected under microscope at a magnification of ×40–100. Monogeneans were picked off the gill scrapings alive, placed under a coverslip and fixed in ammonium picrate solution.

Results and discussion

Infection of fish by *Dactylogyrus* spp. in different fish farms is shown in Table I. In the majority of cases host-specific parasites were found, species which commonly infect European and Asian cultured fishes (Bauer et al., 1981; Gussev, 1985; Molnár and Szakolczai, 1980). *Dactylogyrus vastator* infected three fishes, namely *Cyprinus carpio*, *Carassius auratus* and *Carassius auratus gibelio*. In goldfish, besides the typical *D. vastator* specimens (Fig. 1) parasites resembling *D. vastator* in morphology but having anchors about half the size of the normal specimens (Fig. 2) were commonly found. Determination of the taxonomic position of this *Dactylogyrus vastator forma minor* needs further studies. A similar phenomenon was observed by Sidorov (1956) who found *D. alatus* in *Alburnus alburnus* and *D. alatus forma maior* in *Leuciscus idus*. Two *Dactylogyrus* spp. were found on the gills of the bighead. They were identified as *D. nobilis* and *D. aristichthys*. The copulatory complex of the latter, however, resembled also *D. taihuensis* Long et Lee, 1960, which suggests that the validity of the later species might be questioned. The frequent occurrence of *Dactylogyrus sahuensis* also deserves special attention, as so far this carp parasite has been found only in China (Ling, 1965), in the Far East (Gussev, 1955) and in Hungary (Molnár, 1984). During the short 4-month period of its rearing in fish ponds *Rutilus frisii kutum* was found to be infected by two species of

Table I

Dactylogyrus spp. from Iranian cultured fishes

Dactylogyrus spp.	Hosts	Fish farms				
		Sangar	Sames-kandeh	Esfahan	Khozes-tan	Tehran
Dactylogyrus anchoratus (Dujardin, 1845)	*Cyprinus carpio*	+	+		+	+
D. aristichthys Long et Yu, 1958	*Aristichthys nobilis*	+	+			
D. baueri Gussev, 1955	*Carassius auratus*					
	Carassius auratus gibelio	+				
D. dulkeiti Bychowsky, 1936	*Carassius auratus*	+				+
D. extensus Mueller et Van Cleave, 1932	*Cyprinus carpio*	+	+	+	+	
D. formosus Kulwiec, 1927	*Carassius auratus*					
	Carassius auratus gibelio	+				
D. hypophthalmichthys Achmerov, 1952	*Hypophthalmichthys molitrix*	+	+			+
D lamellatus Achmerov, 1952	*Ctenopharyngodon idella*	+	+			
D. nobilis Long et Yu, 1958	*Aristichthys nobilis*	+	+			+
D. sahuensis Ling, 1965	*Cyprinus carpio*		+			
D. vastator Nybelin, 1924	*Cyprinus carpio*					
	Carassius auratus gibelio	+	+			+
D. vastator forma minor	*Carassius auratus*	+				+
D. frisii Bychowsky, 1933	*Rutilus frisii kutum*	+	+			
D. rarissimus Gussev, 1966	*Rutilus frisii kutum*	+	+			

Dactylogyrus, *D. frisii* and *D. rarissimus*. Both species were found also in older fish living in the Caspian Sea and in the rivers flowing into it. All *Dactylogyrus* spp. found proved to be new for Iran.

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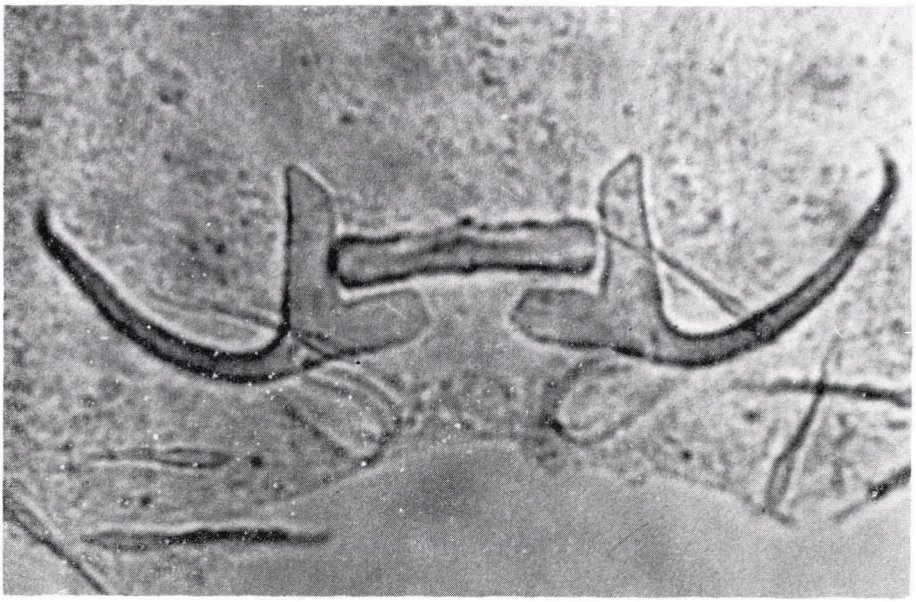

Fig. 1. Anchors and hooks of a typical *Dactylogyrus vastator* specimen from common carp. ×1000

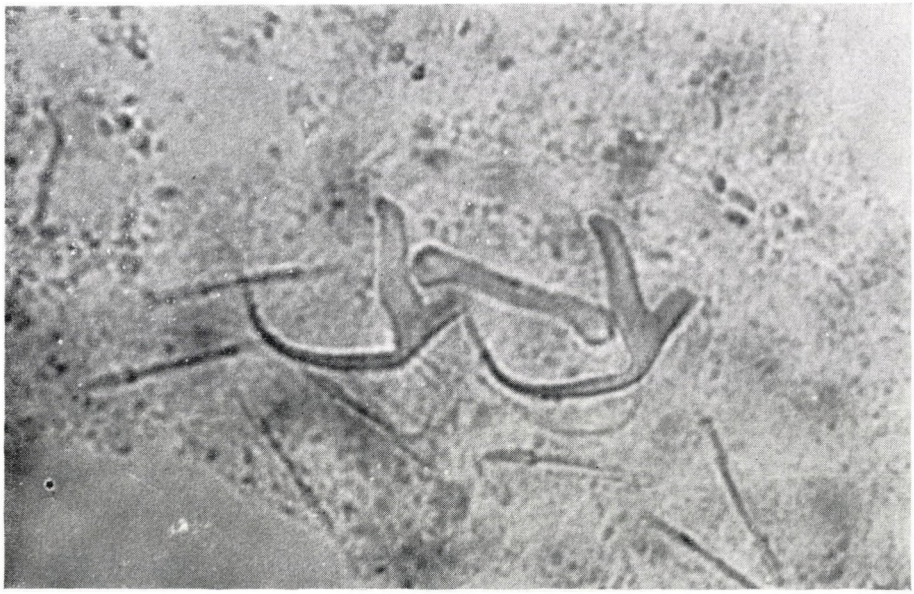

Fig. 2. Anchors and hooks of *Dactylogyrus vastator forma minor* from goldfish. ×1000

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EFFECT OF BENZIMIDAZOLES ON AMINO ACID METABOLISM IN *TRICHURIS GLOBULOSA*

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L-aspartic acid C_{14}, L-alanine C_{14} and L-leucine C_{14} uptake by *Trichuris globulosa* was found to be a non-linear function of time and limiting substrate concentration. The uptake was rapid initially but achieved steady state possibly owing to the saturation of transport loci. Linear transformations of substrate saturation kinetics by Lineweaver–Burk plots of L-aspartic acid C_{14}, L-alanine C_{14} and L-leucine C_{14} gave K_t values of 6.8×10^3 μM, 3.4×10^3 μM and 6.06×10^3 μM and J_{max} of 0.769 μmoles/mg dry weight/min, 10 μmoles/mg dry weight/min and 0.285 μmoles/mg dry weight/min, respectively. The presence of benzimidazole drugs, thiabendazole and fenbendazole, markedly inhibited the uptake of amino acids at concentrations which did not affect the motility of the parasite. The amino acid transport was also found to be pH and temperature dependent. The uptaken amino acids were readily metabolized into different tissue fractions. Thiabendazole and fenbendazole significantly inhibited the incorporation of the three amino acids into the nematode's total protein fractions and trichloroacetic acid soluble fractions. These drugs also decreased the amount of radiocarbon of ^{14}C-amino acids incorporated into CO_2.

Keywords: *Trichuris globulosa*, amino acid uptake, effect of benzimidazoles.

It is generally believed that in helminth parasites only the carbohydrates undergo active metabolism and generate energy. However, recent evidence indicates that other compounds like amino acids, lipids and nucleotides also have a major role in the metabolism as well as synthesis of body constituents. Studies on *Ascaridia galli* have demonstrated that a number of amino acids are absorbed and catabolized at appreciable rates (Singh et al., 1983; Singh and Srivastava, 1983), although relatively little information exists about the role of amino acids in the intermediary metabolism of parasitic helminths (Barrett, 1981).

Benzimidazole drugs have been used as effective anthelmintic compounds, although their modes of action are far from being understood (Borgers et al., 1975*a*). No attempt has been made so far to explore the possibility that these drugs could possibly effectively block the uptake of essential nutrients like hexoses or amino acids and inhibit their transport, resulting in death. The purpose of the present study was to get experimental evidence on the mode of action of thiabendazole and fenbendazole, two benzimidazole derivatives, with regard to amino acid transport and kinetic characterization of amino acid metabolism in *Trichuris globulosa* (v. Linstow), an intestinal parasitic nematode of goats.

Materials and methods

Adult *T. globulosa* were collected from the large intestine of goats *(Capra hircus)* procured from local abattoirs. The nematodes were expeditiously washed in Hanks' balanced salt solution to remove intestinal debris and adhering material.

The nematodes were incubated in 4 ml of incubation medium at 37 °C under aerobic conditions in the presence of 1 μci of L-aspartic acid C_{14} (specific activity = 192 mci/m mole), L-alanine C_{14} (specific activity = 144 mci/m mole) and L-leucine C_{14} (specific activity = 288 mci/m mole) (Bhabha Atomic Research Centre, Bombay, India). Incubation media were made in Krebs–Ringer saline containing 25 mM Tris-HCl buffer at pH 7.4 (KRT of Read et al., 1963) except that maleate had been replaced by HCl since it was shown to be toxic in metabolic studies (Webb, 1966). In experiments in which the effects of thiabendazole and fenbendazole were studied, the drugs dissolved in 0.1% dimethyl sulphoxide (DMSO) were added to the incubation media at a final concentration of 100 μmoles. Control incubations received the same volume of 0.1% DMSO.

In order to minimize the effects of diurnal variation, experiments were performed every day at 10 a. m. The nematodes were randomly allocated into groups of 3–4 worms, each group constituting a single sample. After an incubation of 30 min the incubation medium was removed rapidly by aspiration. Nematodes were rinsed thoroughly in 3×10 ml changes of cold KRT, blotted on coarse filter paper and kept in 2 ml of 70% ethanol overnight. The extracted carcasses were dried overnight at 80 °C in preweighted aluminium foil pans and weighed. Aliquots of incubation medium and ethanol extracts of the worms were counted in a liquid scintillation spectrometer (Beckman Instrument) using methanolic toluene and phosphorus (4 g 2.5-Diphenyloxazol (PPO) + 500 mg 1,4-Di-2 (phenyloxazolyl)-benzene (POPOP)/700 ml toluene + 300 ml methanol) as the liquid scintillant.

Uptake velocities were calculated from specific activity of the media and total activity in the worm extracts and expressed as μmoles substrate absorbed/mg ethanol extracted dry weight/min. For these, the CPM/sample were converted to specific activity by comparison with standards and employing appropriate calculations (Read et al., 1963). All determinations were the means of 3–4 replicates with standard deviation. Data for the uptake of amino acids (V) as function of substrate concentration were examined using plots of each of three linear transformations of the Michaelis–Menten equation; the double reciprocal plot of Lineweaver–Burk, S/V versus S plot of Woolf and V versus V/S plot of Eadie–Hofstee (Siegel, 1976). Apparent J_{max} (maximum uptake activity) and K_t (substrate affinity constant) values were calculated from the linear transformations. However, the data were more appropriate as Eadie–

Hofstee plot, rather than the more widely used Lineweaver–Burk plot, since this was least sensitive to random experimental variations, yet most sensitive to systematic deviation from Michaelis–Menten kinetics (Starling and Fischer, 1975).

The procedure of Crabtree et al. (1977) was followed to extract cold trichloroacetic acid (TCA) soluble fraction, lipids, RNA, DNA and proteins in the incubated parasites. The complete sequence of isolation is as follows: In 1 ml of 10% tissue homogenate, 2.5 ml 10% TCA was added and centrifuged for 10 min at 3,000 × *g* 4 °C. Supernatant I was removed, 2.5 ml of 10% TCA was added to the pellet again and centrifuged at the same rate. Supernatant II was added to supernatant I, these constituting the TCA soluble fraction. Then 5.0 ml of 95% ethanol was added to the pellet and centrifuged at 3,000 × *g* for 10 min. Supernatant III contained the lipids. The step was repeated by adding 5.0 ml of ethanol: ether (3 : 1) and supernatant IV was added to it. Then 2.0 ml 1N KOH was added to the pellet which was incubated at 37 °C for 20 h. It was then neutralized with 6N HCl and 2.0 ml 5% TCA was added and centrifuged at 3,000 × *g* for 10 min. Supernatant V contained RNA while the pellet constituted proteins and DNA. To the pellet, 2.0 ml of 5% TCA was added and incubated for 15 min at 90 °C and then centrifuged at 3,000 × *g* for 10 min. Supernatant VI contained DNA. The step was repeated once again. Supernatants VI and VII were pooled and assayed for DNA. The pellet was dissolved in 1N KOH, incubated at 85 °C for 15 min and brought up to 10 ml with distilled water: this was accepted as the protein fraction. A definite amount of each fraction was shifted to scintillation vials for counting.

To determine the amount of radioactivity contained in glycogen after incubation of *T. globulosa* in the amino acids uniformly labelled with ^{14}C, the following procedure was used: 5 mg non-radioactive glycogen (to serve as carrier) 0.3 ml 10% Na_2SO_4 and 2 volumes of 100% ethanol were added to 3 ml of total TCA soluble fraction. The contents were throughly mixed and, on flocculations, centrifuged at 1,00 × *g* for 10 min. The supernatant was decanted into a separate test tube and the pellet was washed with 1 ml of 100% ethanol and recentrifuged. The supernatant was decanted and added to the previous one and the pellet was dried with gentle heat. The pellet contained all the glycogen and was then hydrolysed with 1 ml of 2N H_2SO_4 at 100 °C for 1 h. A 0.2 ml aliquot of hydrolysate was pipetted out into a scintillation vial, neutralized with 0.2 ml hyamine ×10, dissolved in 10 ml of liquid scintillation counting mixture as mentioned earlier, and radioactivity was determined.

To obtain the counts of ^{14}C-incorporated CO_2, a Warburg-type flask was used. 10% KOH was placed in the central well and the nematodes and incubation medium containing radioisotope in the flask. The flask was tightly stoppered and kept in shaking water bath for 90 min. KOH which absorbed CO_2 formed by metabolism was removed from the central well with a filter paper

piece which was placed into a scintillation vial. After adding liquid scintillant which extracted all the radiocarbon from the filter paper, counts were taken.

Results

The rate of absorption of L-aspartic acid C_{14}, L-alanine C_{14} and L-leucine C_{14} by adult *T. globulosa* was rapid for the initial 30 min and then achieved steady state with time (Fig. 1). From this study, experimental incubations of 30 min were considered optimum for further studies. The rate of uptake of amino acids was inhibited in the presence of thiabendazole and fenbendazole (Fig. 1/A-C). The absorption of amino acids was also found to be a non-linear function of limiting substrate concentration (Fig. 2). Linear transformations of the data gave a straight line in Lineweaver–Burk and Eadie plots (inset of Fig. 2/A-C). Thiabendazole and fenbendazole inhibited the uptake of all the three ^{14}C-amino acids as a higher K_t (lower substrate affinity constant) resulted in the presence of these drugs compared to the control, while the J_{max} was unaltered.

Amino acid absorption was found to be dependent on pH and temperature. Uptake of L-aspartic acid C_{14}, L-alanine C_{14} and L-leucine C_{14} was optimum at pH 7.6, 8.4 and 9.2, respectively (Fig. 3). The transport was also affected by changing the temperature. The rate of uptake was increased with temperature up to 50–60 °C (Fig. 4).

L-aspartic acid C_{14} and L-alanine C_{14} were absorbed rapidly as compared to L-leucine C_{14}. The uptaken amino acids were mainly incorporated into proteins. L-leucine C_{14} absorbed by *T. globulosa* contributed to protein synthesis to a higher degree than L-aspartic acid C_{14} and L-alanine C_{14}. Fifty % of absorbed ^{14}C from L-leucine C_{14} was traced in proteins, as compared to L-aspartic acid C_{14} and L-alanine C_{14} where only 30% and 35% radioactive carbon was obtained in the protein fraction, respectively. L-aspartic acid C_{14}, L-alanine C_{14} and L-leucine C_{14} contributed almost to the same degree to the synthesis of DNA and RNA, that is, to DNA 3%, 2% and 3.5% and to RNA 10%, 7% and 8.5%, respectively. The amount of radiocarbon incorporated into CO_2 was also determined: it was 10%, 12% and 15% for L-aspartic acid C_{14}, L-alanine C_{14} and L-leucine C_{14}, respectively. Unbound glycogen and lipids showed very little amount of incorporated carbon.

In the presence of thiabendazole and fenbendazole, the incorporation of amino acids into different tissue fractions was inhibited. The inhibition was significant in the case of the TCA soluble fraction, protein fraction and also CO_2 evolved in an *in vitro* incubation.

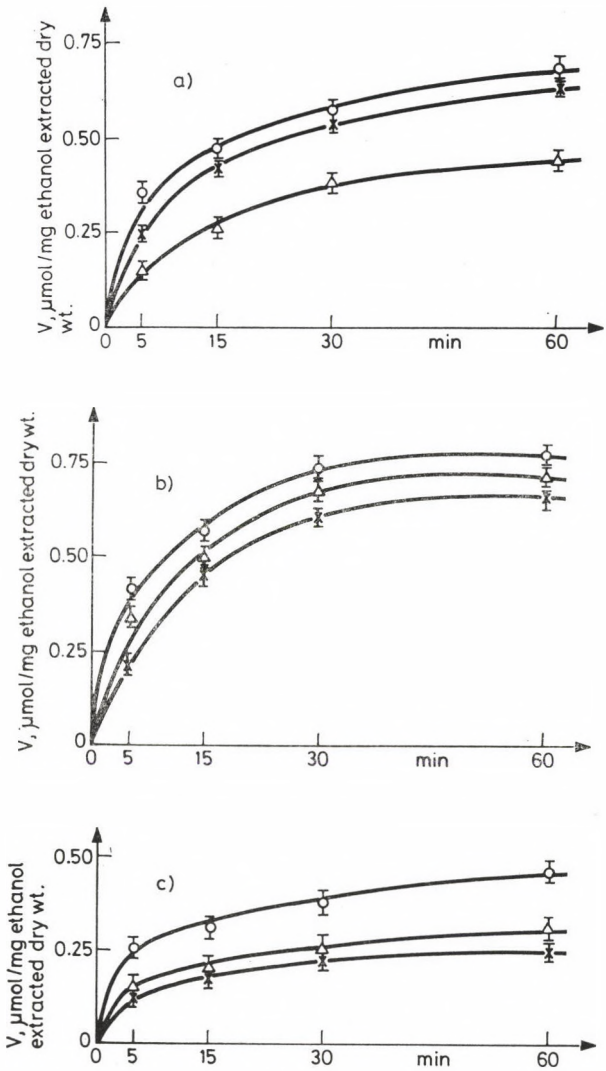

Fig. 1. Time-course study of the uptake of (A) ^{14}C-aspartic acid, (B) ^{14}C-alanine, and (C) ^{14}C-leucine by *Trichuris globulosa* in the absence (○–○–○) and presence of thiabendazole (△–△–△) and fenbendazole (×–×–×). V is uptake velocity. The results are the mean ±SD of 4 observations. Longitudinal bars represent SD

Discussion

The basic mechanism of amino acid transport in *T. globulosa* appears to be the same as the glucose transport in this particular nematode (Jasra et al., 1989) and also in *Ascaridia galli* (Aggarwal et al., 1989) as reported. The process may be the same as postulated for higher animals (Crane, 1965) in that

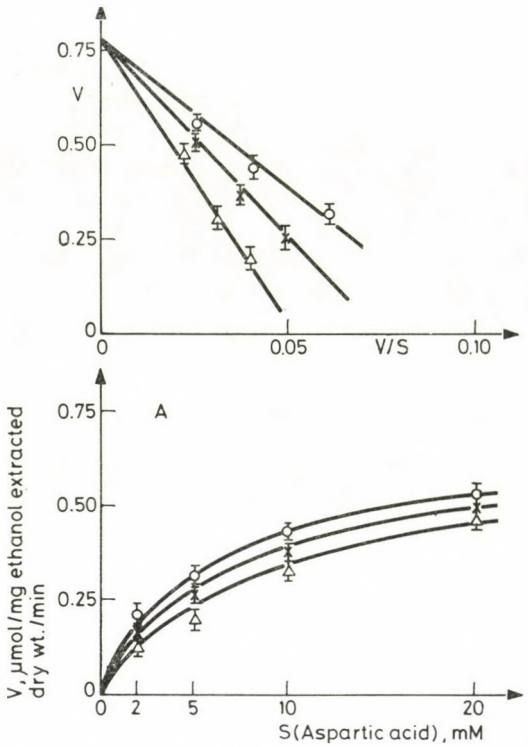

Fig. 2A

Fig. 2. The velocity of uptake (V) of (A) ^{14}C-aspartic acid, (B) ^{14}C-alanine, and (C) ^{14}C-leucine by *Trichuris globulosa* in the absence (○–○–○) and presence of thiabendazole (△–△–△) and fenbendazole (×–×–×) as the function of substrate concentration (S). The results are the mean ±SD of 4 replicates. Longitudinal bars represent SD. Insets of Figs 2/A, B and C: Eadie–Hofstee plots of uptake of the respective ^{14}C-amino acids

the influx is accumulative, mediated and dependent on time until the transport loci are saturated. Asch and Read (1975) reported that the uptake of alanine and glycine (neutral amino acids) and glutamic acid and aspartic acid (acidic amino acids) occurs both by diffusion and by mediated system in *Schistosoma mansoni* (blood fluke). On a molar basis the rate of incorporation of acidic amino acids is lower than that of neutral ones (Jaffe and Doremus, 1970). The data, when plotted according to the Eadie–Hofstee linear transformation plot, suggest that the uptake of amino acids is mediated by transporter proteins. The transport system of amino acids is also pH and temperature dependent. Thiabendazole and fenbendazole were found to inhibit the amino acid uptake by *T. globulosa* as a higher K_t value (lower substrate affinity) was observed while the J_{max} (maximum velocity) was not changed. Similar results have been obtained by Ahmed and Nizami (1987) on other helminths. Mebendazole was reported to impair the absorptive functions of helminths (Verheyen et al., 1976) and to cause exten-

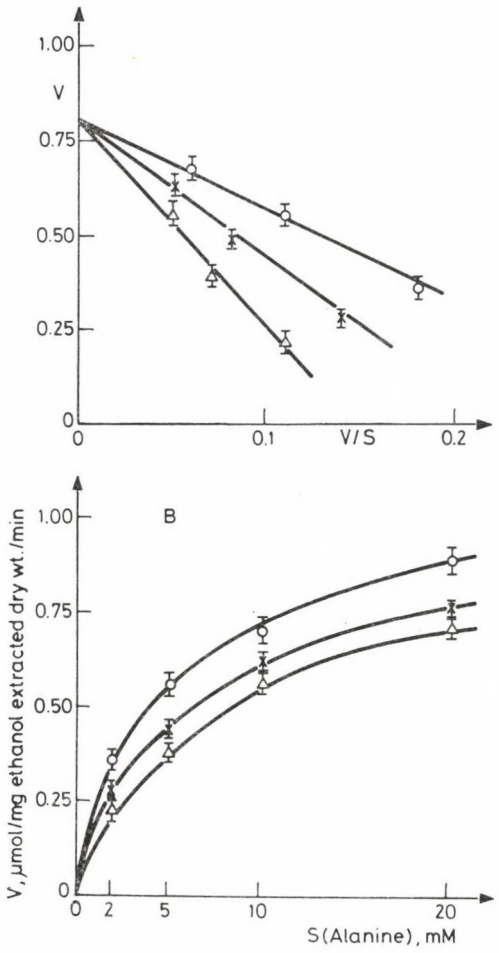

Fig. 2B

sive ultrastructural damage to the absorptive surfaces of nematodes and cestodes (Borgers et al., 1975*b*). The inhibition of amino acid metabolism mainly contributes toward the inhibition of synthesis of body constituents. Thiabendazole and fenbendazole seem to block the binding, solubilization and utilization of substrate by the transporter proteins.

Like other nematodes including *Ascaris lumbricoides* (Pollack and Fairbairn, 1955), *Setaria cervi*, *Litomosoides carinii* and *Dipetalonema viteae* (Singh and Srivastava, 1984), *T. globulosa* transports and transaminates alanine and aspartate most profoundly. *T. globulosa* also shares this property with other parasites such as *Fasciola hepatica* (Daugherty, 1952).

The amino acids taken up by *T. globulosa* undergo metabolism and are possibly utilized largely in the synthesis of proteins. Nollen (1968) reported that

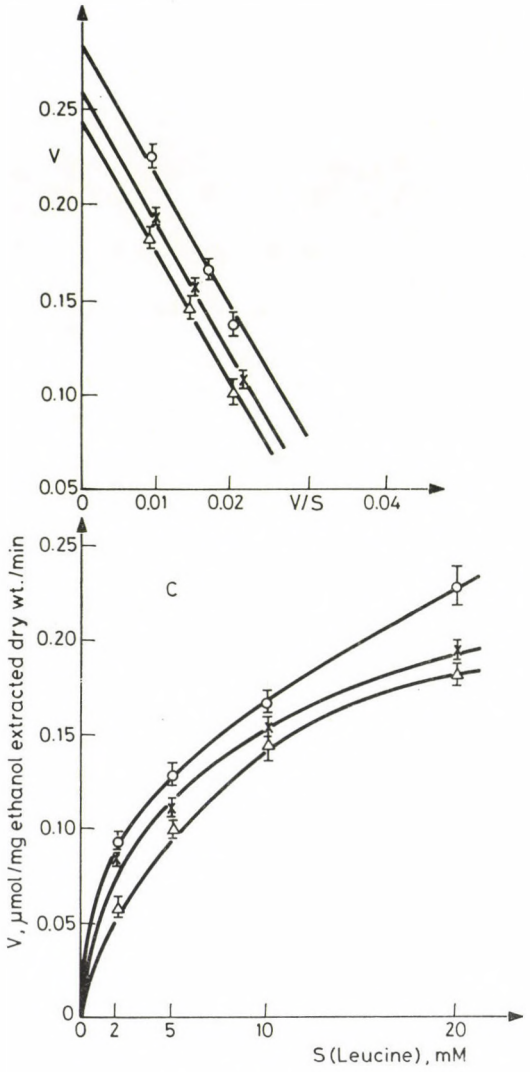

Fig. 2C

L-leucine becomes incorporated in constituents of protein in trematodes. Similar reports have been given by Haynes and Taylor (1968) on *Taenia crassiceps*.

A part of the absorbed amino acids also incorporated into DNA, RNA, glycogen, lipids, and CO_2 in *T. globulosa* as in other organisms such as epimastigotes. In *T. globulosa* very small amounts of amino acids were incorporated into DNA and glycogen while the RNA and CO_2 fraction showed a fair amount of incorporated radiocarbon from ^{14}C-amino acids. This result is supported by the findings of Jaffe and Doremus (1970). The benzimidazole drugs thiabendazole

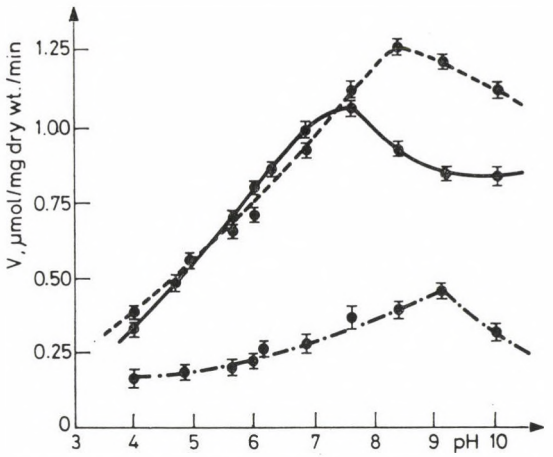

Fig. 3. Effect of pH on the influx of ^{14}C-aspartic acid (———), ^{14}C-alanine (– – –) and ^{14}C-leucine (–·–·–) by *Trichuris globulosa*. Each point is the mean ±SD of 4 replicates. V is uptake velocity. Longitudinal bars represent SD

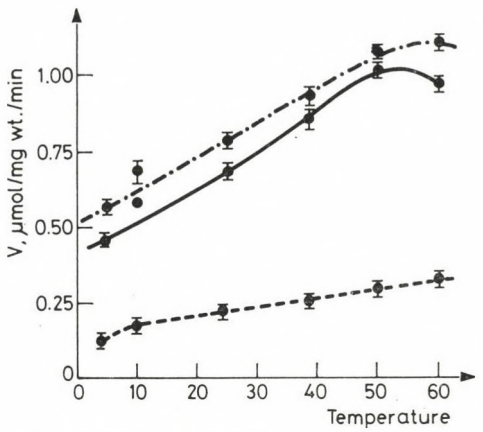

Fig. 4. Effect of temperature on the absorption of ^{14}C-aspartic acid (———), ^{14}C-alanine (– – –) and ^{14}C-leucine (–·–·–). Each point is the mean ±SD of 4 replicates. V is uptake velocity. Longitudinal bars represent SD

and fenbendazole impair the amino acid metabolism by inhibiting uptake and incorporation into different fractions, mainly into the TCA-soluble fraction, which may provide some clue to the mode of action of this group of anthelmintics.

Table I

Incorporation of radioactivity of amino acids into various tissue fractions of *Trichuris globulosa*

Isolated fraction	% * of incorporated ^{14}C-aspartic acid			% of incorporated ^{14}C-alanine			% of incorporated ^{14}C-leucine		
	Normal	Thiabendazole-treated	Fenbendazole-treated	Normal	Thiabendazole-treated	Fenbendazole-treated	Normal	Thiabendazole-treated	Fenbendazole-treated
TCA-soluble	40±2	30±3	30±4	45±4	30±3	33±3	50±5	40±3	45±4
Proteins	30±2	20±2	25±3	35±4	25±3	28±4	45±5	28±3	30±5
RNA	10±1	7±1.5	8±1	7±2	4±0.3	5.5±0.9	9±1	5±1	7±1.5
DNA	3±0.2	1.5±0.3	2.5±0.5	2±0.5	1±0.2	1.5±0.4	2±0.3	1±0.2	1.8±0.5
Lipids	5±0.8	2.5±0.7	3±0.8	6±0.9	3±0.2	4±0.9	4±0.5	2±0.3	2.5±0.5
Glycogen	1.5±0.8	0.5±0.2	0.9±0.2	1.9±0.2	0.8±0.2	1±0.4	1.7±0.5	0.8±0.1	1.2±0.4
CO_2	10±1	8±1	8±2	12±1	8±0.8	9±2	15±2	10±0.8	11±0.9

* The percentage of a particular fraction is calculated by counts per min for that particular fraction and by taking total incorporation of ^{14}C-amino acid/min as 100 counts. Each value is mean ± SD of 3 determinations.

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ASCARIDIA GALLI: EFFECT OF SOME ANTHELMINTICS ON AMINO ACID UPTAKE AND MACROMOLECULAR SYNTHESIS *IN VITRO*

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L-(U-^{14}C) aspartic acid, L-(U-^{14}C) alanine and L-(U-^{14}C) leucine uptake by *Ascaridia galli* was found to be a non-linear function of time and limiting substrate concentration. The uptake was rapid initially but achieved steady state thereafter, possibly owing to the saturation of transport loci. Linear transformations of substrate saturation kinetics by Lineweaver–Burk plots of L-(U-^{14}C) aspartic acid, L-(U-^{14}C) alanine and L-(U-^{14}C) leucine gave K_t values of 4.76, 3.03 and 2.0 mM and J_{max} of 5.0, 3.57 and 2.08 m moles/100 mg dry weight/2 min, respectively. Dl-tetramisole and l-tetramisole (levamisole) inhibited the uptake of amino acids. The uptaken amino acids were readily metabolized into different tissue fractions. Dl-tetramisole and levamisole significantly inhibited the incorporation of the three amino acids into the nematode's total protein, RNA and lipid fractions in an *in vitro* incubation system.

Keywords: *Ascaridia galli*, amino acid uptake, macromolecular synthesis, tetramisole

It is generally believed that while in helminth parasites only carbohydrates undergo active metabolism and generate energy, other compounds like amino acids, lipids and nucleotides participate largely in the synthesis of body constituents. Studies on *Ascaridia galli* (Schrank), an intestinal nematode of fowl, have, however, clearly demonstrated that a few amino acids are also catabolized at appreciable rates (Singh et al., 1983*a*, *b*; Singh and Srivastava, 1983) and some of them yield ATP too (Singh et al., 1983*c*). Relatively little information exists, however, about the role of amino acids in the intermediary metabolism of parasitic helminths (Barrett, 1981).

Dl-tetramisole and levamisole are broad-spectrum anthelmintics potent against the common worm infestations of birds, cattle and man. Considerable information has now accumulated indicating that the levoisomer is more active than the dl-compound (Kates et al., 1971). Both dl-tetramisole and levamisole have a dual inhibitory action on nematodes by having a direct stimulating effect on muscular contraction (paralytic) as well as an inhibitory effect upon the fumarate reductase system (Bossche, 1972; Bossche and Janseen, 1967). In order to further understand the mode of action, the present report deals with the effect of these anthelmintics on amino acid uptake and macromolecular synthesis in *A. galli*.

Materials and methods

Adult *A. galli* were collected from the intestine of fowl procured from local poultry farms, and thoroughly washed in saline to remove intestinal debris and adhering materials. All incubations were done in Krebs' Ringer solution, containing 25 mM tris (hydroxymethyl amino methane)-HCl buffer at pH 7.4 (KRT of Read et al., 1963) at 37 °C.

Approximately 500 mg of worms were placed in each 25 ml Warburg flask containing 4.0 ml of incubation media having 1.0 μci of radioactive amino acid. To measure the formation of respiratory $^{14}CO_2$, 0.3 ml of 10% KOH (carbonate free) was placed in the center well of the Warburg flask containing filter paper. After an incubation of 90 min, the filter paper was carefully removed from the center well and immersed directly into the counting vial containing scintillation fluid. The scintillation fluid for counting the β-emitting radioisotopes contained toluene–methanol, 700 : 300 (v/v), 4 g 2.5-Diphenyloxazol (PPO) and 50 mg 1,4-Di-2-(phenyloxazolyl)-benzene (POPOP). The counting was recorded in a Beckman instrument. The worms were taken out into centrifuge tubes for thorough washing with chilled Krebs' Ringer solution before further use, homogenized in a cold buffer containing 0.15 M NaCl and 0.015 M sodium citrate (pH 7.3), and a 10% homogenate was made. Four ml of this was taken out, supplemented with 2.5 ml cold trichloroacetic acid (TCA, 10%), centrifuged and the supernatant was collected. The precipitate was resuspended 2.5 ml cold TCA (10%) again and the same was repeated. Both supernatants were pooled and called acid (TCA) soluble phosphorus fraction.

To determine the amount of radioactivity in glycogen after incubation of nematodes in the presence of radioactive precursors the following procedure was used: 5 mg nonradioactive glycogen (to serve as a carrier), 0.3 ml of 10% Na_2SO_4 and 2 volumes of 100% ethanol were added to 3 ml of total cold TCA-soluble placed in a conical glass centrifuge tube, and the contents were thoroughly mixed. Upon flocculation, the contents were centrifuged at 1,000 *g* for 10 min. The supernatant was decanted into a test tube and the pellet was washed with 1 ml of 100% ethanol and recentrifuged. The supernatant was decanted and added to the previous tube while the pellet was dried with gentle heating. The procedure removed essentially all the glycogen present in the cold TCA-soluble fraction, as determined by the phenol–sulphuric acid colorimetric method for sugar. The glycogen so isolated was hydrolysed with 1 ml 2N H_2SO_4 at 100 °C for 1 h. After cooling, a 0.2 ml aliquot of the hydrolysate was pipetted into a counting vial, neutralized by the addition of 0.2 ml Hyamine $\times 10$ and dissolved in 5 ml of liquid scintillation counting mixture.

Alcohol was removed from the glycogen-free TCA-soluble fraction by evaporation under pressure. The volume was made up to 2.0 ml with 100% TCA and a 0.2 ml aliquot was removed for liquid scintillation counting.

Lipids, RNA, DNA and proteins were isolated from the TCA-insoluble fraction as described by Crabtree et al. (1977). To the TCA-insoluble fraction, 5.0 ml of 95% ethanol was added, centrifuged at 800 *g* for 10 min at 4 °C, then with 5.0 ml ethanol–ether (3 : 1) and recentrifuged. The combined supernatant solvents were decanted into counting vials, dried and radioactivity counts were taken.

RNA was extracted from the remaining precipitate with 2.0 ml 1N KOH, incubated at 37 °C for 20 h. It was neutralized with 6N HCl, added 200 ml of 5% TCA and centrifuged at 800 *g* for 10 min at 4 °C. The supernatant was taken into a counting vial, dried, scintillation fluid was added, and radioactivity counts were taken. For isolation of DNA, 2.0 ml of 5% TCA was added to the RNA-free precipitate. It was then incubated then at 90 °C for 15 min, centrifuged at 800 *g* for 10 min at 4 °C, and this was repeated twice. The combined supernatant was used for radioactivity counts for DNA. The precipitate remaining after this step was taken to proceed for proteins. To this precipitate 1.0 ml of 1N KOH was added and kept at 85 °C for 15 min. This was made up to 10 ml with water. Aliquots (0.2 ml) of each 10 ml extract of proteins were removed for liquid scintillation counting. The effect of the anthelmintics dl-tetramisole and levamisole on amino acid uptake at different time intervals and macromolecular synthesis were studied by adding the drugs to the standard incubation system at a concentration of 0.04 mM. This concentration was determined on the basis of initial laboratory experiments where it was found that the worms were completely paralyzed. Complete cassation of motility was found with dl-tetramisole after 45 min and with levamisole after 30 min at 37 °C. Radioactive substances used in this work (L- (U-^{14}C)-aspartic acid, sp. act. = 192 mci/m mole; L- (U-^{14}C)-alanine, sp. act. = 144 mci/m mole; L- (U-^{14}C)-leucine, sp. act. = 288 mci/m mole) were obtained from the Bhabha Atomic Research Centre, Bombay, India. Data on the uptake of amino acids as a function of substrate concentration were examined using plots of each of 3 linear transformations of the Michaelis–Menten equation; the 1/V versus 1/S of Lineweaver–Burk, the V versus V/S of Eadie–Hofstee, and the S/V versus S plot of Woelf (Segel, 1976). However, the data are more appropriate as Eadie plot since this plot has been found to be least sensitive to systematic deviation from Michaelis–Menten kinetics (Starling and Fischer, 1975).

Results

The uptake of radiolabelled aspartic acid, alanine and leucine by adult *A. galli* is found to be a non-linear, two-component process as a function of time and also shows substrate saturation kinetics. An initial rapid uptake for the first two minutes is followed by a second lower mode of uptake up to the

next 30 minutes, achieving steady state (Figs 1–3). From this study, experimental incubations of 2 min are chosen as optimal for further studies. The rate of uptake of amino acids was inhibited in the presence of dl-tetramisole and levamisole (Figs 1–3). The uptake of amino acids was also found to be non-linear with respect to external substrate concentration. Linear transformations of the

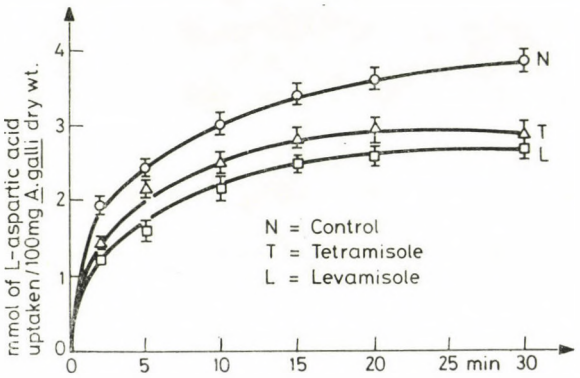

Fig. 1. Time-course of L-(U-^{14}C) aspartic acid in *A. galli* in the presence and absence of drug. Each point is the mean of three determinations. Longitudinal bars indicate SD of the mean

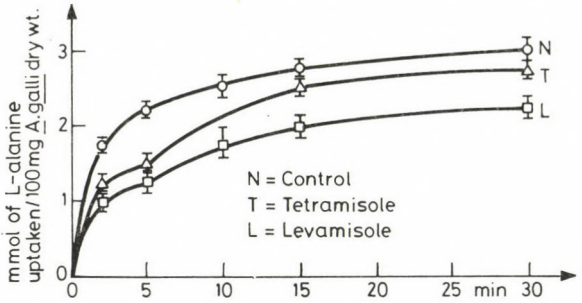

Fig. 2. Time-course of L-(U-^{14}C) alanine in *A. galli* in the presence and absence of drug. Each point is the mean of three determinations. Longitudinal bars indicate SD of the mean

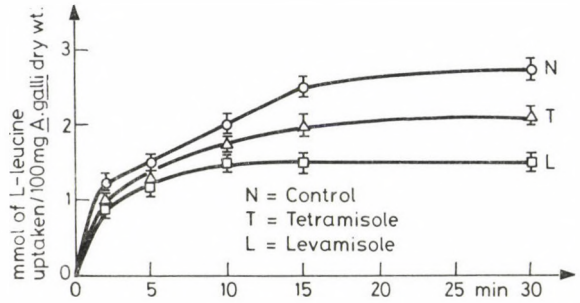

Fig. 3. Time-course of L-(U-^{14}C) leucine in *A. galli* in the presence and absence of drug. Each point is the mean of three determinations. Longitudinal bars indicate SD of the mean

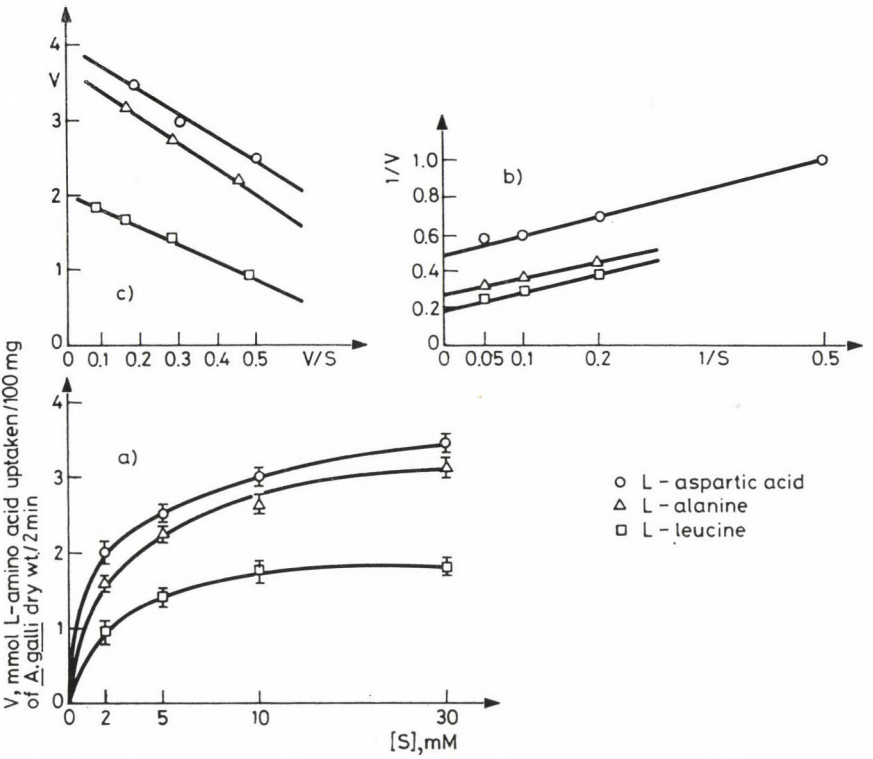

Fig. 4. Influx of L-(U-^{14}C) amino acids in *A. galli* as a function of substrate concentration. V = millimoles of (^{14}C) amino acid absorbed per 100 mg of ethanol extracted dry weight per 2 min. (S): substrate concentration (millimolar). Each point is the mean of three determinations. Longitudinal bars indicate SD of the mean. (b) Lineweaver–Burk plot of 1/V versus 1/S. (c) Eadie plot of V versus V/S

data on the effect of substrate concentration are presented in the form of Lineweaver–Burk and Eadie plot (Figs 4*a*—*c*). The K_t of aspartic acid, alanine and leucine uptake was found to be 4.76, 3.03 and 2.0 mM and the J_{max} was 5.0, 3.57 and 2.08 m moles/100 dry weight/2 min, respectively.

Aspartic acid and alanine were absorbed rapidly as compared to leucine. Table I shows that the parasite produced maximum CO_2 from aspartic acid and alanine but less in the presence of leucine. In percentage terms all the three amino acids contributed almost to the same level to the synthesis of lipid, DNA, RNA, protein, TCA-soluble, glycogen and CO_2 fractions. The DNA incorporated very little amount of radiolabelled carbon. All the three amino acids yielded appreciable amounts of RNA and proteins. The synthesis of $^{14}CO_2$ and TCA-soluble fractions was maximum.

D1-tetramisole and levamisole inhibited the incorporation of radiolabel into different macromolecular fractions. The inhibition was more expressed in

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Table I

Effect of anthelmintics on incorporation of radiolabelled amino acids into different macromolecular fractions of *A. galli*

Radiolabelled amino acid	Anthelmintic	Counts per min recovered 100 mg 90 min into different macromolecular fractions						
		CO_2	TCA-soluble	Glycogen	Lipid	RNA	DNA	Protein
L-(U-^{14}C) aspartic acid	No addition	3200±102 (43.27)	2780±103 (37.50)	250±13 (3.30)	154±28 (2.08)	390±20 (5.20)	70±12 (0.90)	550±30 (7.40)
	Tetramisole	3000±96	2600±101	260±12	114±22	240±40	65±10	402±22
	Levamisole	2900±100	2500±102	265±13	102±24	210±21	65±10	370±20
L-(U-^{14}C) alanine	No additon	2500±96 (39.60)	2500±102 (39.60)	270±10 (4.20)	104±04 (1.65)	350±25 (5.50)	65±08 (1.03)	510±15 (8.09)
	Tetramisole	2445±110	2400±106	273±12	78±10	240±40	62±07	420±28
	Levamisole	2410±120	2335±107	275±30	65±12	210±27	60±13	360±34
L-(U-^{14}C) leucine	No additon	1600±102 (38.08)	1500±95 (36.60)	210±27 (6.12)	104±04 (2.50)	270±20 (6.53)	60±14 (1.46)	350±27 (8.60)
	Tetramisole	1540±100	1400±94	212±12	80±12	210±25	59±10	270±24
	Levamisole	1500±101	1335±92	214±18	70±11	170±14	55±09	250±22

Results are expressed as mean ± SD of 3–4 observations. Values presented in parentheses indicate the percentage incorporation of total radioactivity in the individual fraction

the presence of levamisole as compared to dl-tetramisole. RNA, protein and lipids were inhibited quite significantly. No change in glycogen synthesis was observed in the presence of drugs (Table I).

Discussion

The basic mechanism of amino acid transport in *A. galli* appears to be the same as that of glucose transport in this nematode (Aggarwal et al., 1989). Dryuchenko and Berdyeva (1974) also reported that active transport as well as diffusion are involved in the uptake of amino acids by ascarids. Transcuticular uptake of l-glycine and l-arginine involved both diffusion and carrier-mediated transport in adult female *Brugia pahangi* (Howells et al., 1983). Gray et al. (cit. Uglem and Read, 1973) found mediated uptake of ^{14}C-leucine and ^{14}C-alanine in *Moniliformis dubius*. *A. galli* absorbs l-aspartic acid and l-alanine more rapidly than l-leucine. Ando et al. (1980) studied the uptake of amino acids in microfilariae of *Dirofilaria immitis*. They observed that the quantity of both aspartic acid and leucine decreased considerably in the incubation medium thereby indicating more uptake by the parasite; however, alanine did not show much difference.

The amino acids taken up by *A. galli* undergo metabolism and are utilized largely in TCA-soluble fraction and $^{14}CO_2$ production. Jaffe and Doremus (1970) also found maximum incorporation in TCA-soluble fraction when microfilariae of *D. immitis* were incubated with radiolabelled amino acids. Rate of $^{14}CO_2$ production by *A. galli* was maximum in the presence of alanine and aspartic acid compared to leucine as also found by Singh et al. (1987) for *Ancylostoma ceylanicum*. An appreciable amount of radiolabel was found in RNA and protein fractions. Synthesis of glycogen and lipids was found to be low but DNA shared a very little amount. These results support the findings of Jaffe and Doremus (1970).

Dl-tetramisole and levamisole inhibited the uptake of amino acids and the synthesis of lipids, RNA and proteins. TCA-soluble fraction, DNA and CO_2 were least affected while there was no change in glycogen synthesis. Kaur and Sood (1983) found that dl-tetramisole damages the absorptive surface of adult *Haemonchus contortus in vitro*. Dl-tetramisole inhibited the ATP activity in *H. contortus* which is related to lipid synthesis (Kaur and Sood, 1982). Kapur and Sood (1986) also reported less incorporation of ^{14}C-acetate and ^{14}C-glucose into the lipids on addition of dl-tetramisole in *H. contortus*. Singh et al. (1983*c*) demonstrated that incorporation of ^{32}P into ATP by *Setaria cervi* and *Ascaridia galli* was inhibited in the presence of tetramisole.

Our results indicate that *in vitro* the levoisomer is more effective than the dl-compound. Forsyth (1968) also showed that the levoisomer is more active

against gastrointestinal nematodes of sheep and cattle. Both dl-tetramisole and levamisole affect the macromolecular synthesis, suggesting that secondary to the paralysis of the parasite, a change in macromolecular synthesis may be effected as a consequence of altered energy metabolism.

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CHANGES IN THE LIPID PEROXIDE STATUS OF BROILER CHICKENS IN ACUTE MONENSIN POISONING

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The effect exerted by overdosage of monensin, an ionophore antibiotic, on the lipid peroxide status of broiler chickens was studied. Three-week-old broiler cockerels were given 150 mg monensin/kg body mass through a tube, and the malondialdehyde (MDA) concentration, glutathione peroxidase (GSH-Px) and catalase activity of the liver and breast muscle, and MDA concentration and GSH-Px activity of the blood plasma were determined.

Liver MDA and catalase values rose rapidly and significantly during the experimental period. GSH-Px activity initially decreased, then tended to rise. Blood plasma and breast muscle variables did not change during the experiment.

Acute monensin poisoning induced substantial enhancement of lipid peroxidation processes in the liver, while it did not appreciably affect the lipid peroxide status of the blood plasma and breast muscle.

The role of the observed phenomenon in the rather complex pathogenesis of monensin poisoning is not known sufficiently. Further studies are needed to elucidate the problem.

Keywords: Monensin poisoning, broiler chicken, lipid peroxidation, malondialdehyde, catalase, glutathione peroxidase

The effect of monensin on cells, similarly to some protozoa and bacteria, is based on a cation–proton exchange during which monensin, owing to the formation of a lipid-soluble complex, is able to introduce sodium, enclosed in its molecule, into the cell through the double lipid layer of the cell membrane. During reverse transport the ionophore molecule transports hydrogen ion instead of metal ion ("protonated form"). Ionic dysequilibrium and transient acidosis thus developing on the outer surface of the cell membrane are counterbalanced by influx of H^+ ions and efflux of K^+ ions, and the cell loses potassium. Elevated intracellular sodium concentration brings about a secondary increase in calcium concentration, partly due to the release of bound calcium present in the mitochondria and sarcoplasmic reticulum, partly because of reduced calcium outflow from the cells (Gräfe et al., 1984; Bergen and Bates, 1984).

As a consequence of the biochemical processes outlined above, uptake of toxic doses of monensin causes mitochondrial damage and activates cellular

phospholipase and proteolytic enzymes, primarily in the myocardium and striated muscle.

Simultaneously with or subsequent to these changes, ionophores (e.g. monensin) can produce numerous metabolic disturbances either directly or indirectly. They can alter the metabolism of lipids, oxidation of arachidonic acid and, as a result, biosynthesis of prostaglandin, the cAMP level of cells, protein phosporylation, oxidative metabolism, and protein, DNA and RNA synthesis (Gräfe et al., 1984). In monensin poisoning of farm animals primarily the myocardium and striated muscle suffer damage. According to certain authors, however, capillaries, hepatocytes and other cells may also become affected, depending on the animal species.

In some species gross lesions and microscopic changes demonstrable in ionophore antibiotic poisoning (Zenker's degeneration of striated muscle and myocardium) resemble pathomorphological changes caused by vitamin E and selenium deficiency so closely that differential diagnosis is rather difficult (Sályi et al., 1988). This resemblance, and the finding that pretreatment with vitamin E and selenium reduces the severity of monensin toxicosis (Van Vleet et al., 1983), suggests that primary or secondary damage, i.e. oxidation and peroxidation, of lipids building up biological membranes may have a role in the very complex pathogenesis of monensin poisoning.

Monensin is metabolized into degradation products of reduced biological activity primarily in the liver, and the metabolic end-products are excreted in the bile (Donoho, 1984). Certain factors affecting monensin toxicity are supposed to be related to hepatic function or dysfunction.

The influence of monensin poisoning on lipid peroxidation processes and on certain variables indicative of the status of the biological antioxidative defence mechanism was studied in animal experiments.

Materials and methods

Acute poisoning was induced in 3-week-old broiler cockerels (Hybro) by oral treatment with 150 mg monensin/kg body mass, given in the form of Elancoban 100 premix. The birds' feed contained no other anticoccidials or drugs. Before monensin treatment and at 4, 20, 44 and 68 h thereafter 10 treated and 10 control chickens were killed by bleeding. The blood plasma, liver and breast muscle (m. pect. superf.) samples were frozen and stored at —18 °C until processed. Before the chemical determinations the organ samples were homogenized in cold (+4 °C) saline (1 : 9) with a Potter–Elvehjem homogenizer. Malondialdehyde concentration, which indicates the degree of lipid peroxidation, was determined in the intact homogenate by the 2-thiobarbituric acid reaction (Mihara et al., 1980). Enzyme activities were determined in the

supernatant obtained by centrifuging the homogenate at 10,000 *g* for 20 min at +4 °C. Catalase (E.C. 1.11.1.6.) activity was determined by the method of Beers and Sizer (1952) in the presence of hydrogen peroxide substrate. Glutathione peroxidase (E.C 1.11.1.9.) activity was determined by a direct end-point assay as described by Matkovics et al. (1988), in the presence of reduced glutathione and cumen-hydroperoxide substrates. Enzyme activities were related to 1 g protein content of the supernatant which was determined using Folin's phenol reagent (Lowry et al., 1951).

The results were analysed statistically by Student's two-sample *t* test.

Clinical observations

Clinical symptoms appeared very soon after monensin administration. *Ninety min* after monensin treatment the majority of chickens were sitting or lying on their sides with outstretched legs, and the non-recumbent birds also moved unsteadily and showed signs of depression. *At 4 h*, all treated chickens were recumbent, sometimes made swimming movements with outstretched legs, kept craning their neck but were unable to lift their head. *At 20 h* after monensin treatment, the birds' clinical status tended to improve; however, only few chickens were able to stand and walk. The birds excreted watery faeces. *At 44 h*, about half of the chickens were lying with legs outstretched or drawn under themselves. Despite pronounced drowsiness, the birds ate and drank when placed within reach of the feeders or waterers. About 20% of the chickens walked normally but sat down readily. The others had a staggering, unsteady gait, walked with flexed joints and resting on drooping wings. *At 68 h*, the great majority of the chickens could stand up but soon sank to the ground.

Only 3 out of the 50 chickens died (on the 2nd and 3rd day of treatment). No pathognostic lesions were seen at necropsy.

Results

During the 68-hour period of study, no marked differences were demonstrable in the GSH-Px activity of the liver (Fig. 1) and blood plasma between birds with experimentally induced acute monensin poisoning and the control chickens.

Malondialdehyde (MDA) concentration of the blood plasma was similar in the experimental and control chickens (Fig. 2). In contrast, MDA (more exactly: thiobarbituric acid reactive compound) concentration of the liver homogenate of experimental birds moderately exceeded that of the control birds already in the 4th hour, and subsequently this difference became highly significant (Fig. 3).

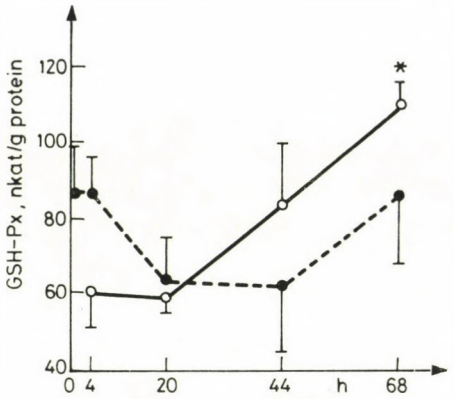

Fig. 1. Changes in glutathione peroxidase (GSH-Px) activity of the liver. Broken line: control group; continuous line: group treated with 150 mg monensin/kg body mass per os (* : $P < 0.05$)

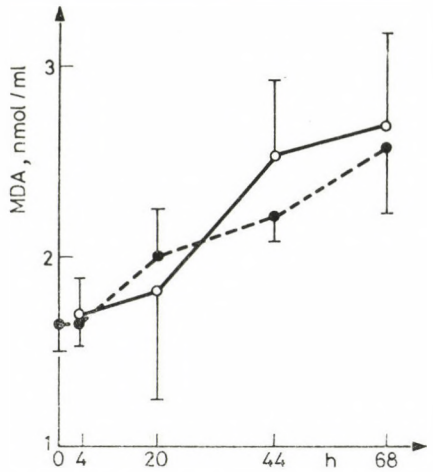

Fig. 2. Changes in malondialdehyde concentration of the blood plasma. Broken line: control group; continuous line: group treated with monensin

The changes in catalase activity of the liver tissue of monensin-intoxicated chickens were similar both in tendency and degree to those in MDA concentration (Fig. 4).

MDA concentration and GSH-Px as well as catalase activity of breast muscle homogenates showed high variation as compared to respective values of the liver in both the experimental and the control group. Disregarding differences of low significance ($P < 0.05$) found in a few cases, no characteristic differences were demonstrable. By comparing the mean values (and disregarding catalase activities measured at 44 h because of the extremely high standard deviation), all three variables (MDA, GSH-Px and catalase) were, even if only slightly (by 5–45%), lower in the muscle tissue of the treated chickens.

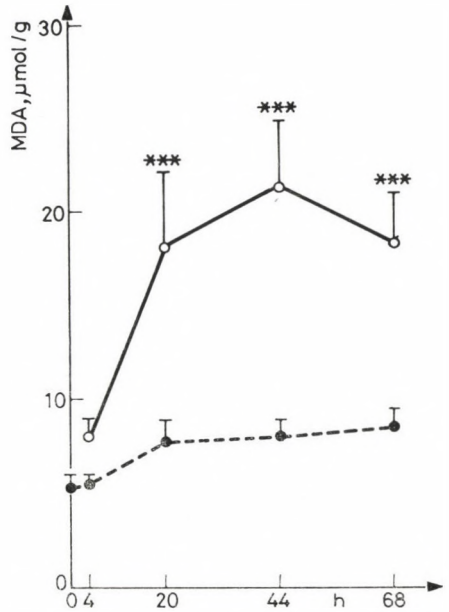

Fig. 3. Changes in malondialdehyde concentration of the liver tissue. Broken line: control group; continuous line; group treated with monensin (*** : $P<0.001$)

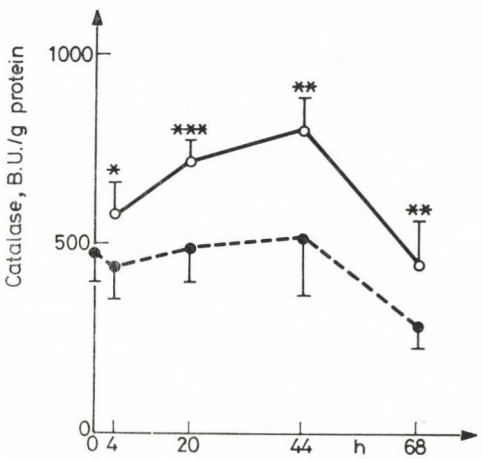

Fig. 4. Changes in catalase activity of the liver tissue. Broken line: control group; continuous line: group treated with monensin (* : $P<0.05$; ** : $P<0.01$; *** : $P<0.001$)

Discussion

In this experiment, marked elevation of the catalase activity and malondialdehyde concentration of the liver tissue indicated a significant enhancement of lipid peroxidation processes in the liver. In harmony with our findings, Dworschák and Prohászka (1986) reported a significant rise in serum and liver malondialdehyde concentration during prolonged feeding of a diet containing 120 mg salinomycin/kg feed (i.e. double of the preventive dose).

For a better understanding of the mechanism of action of ionophore poisoning it would be important to determine whether the enhancement of lipid peroxidation is a primary or a secondary process. Hepatic MDA concentration begins to change rather quickly (in less than 4 h). Despite this fact, the increase of lipid peroxidation is considered, at least for the time being, a secondary process, especially if the very rapid onset of clinical signs is taken into account. It is possible that compounds formed or released during muscle fibre damage are transported by the circulation to the liver where they activate the detoxifying mechanism. The activated detoxification processes, in turn, may give rise to compounds indicative of lipid peroxidation (e.g. malondialdehyde) and activate the antioxidative defence mechanism.

GSH-Px enzyme activity of the liver tissue underwent a moderately significant decrease at the beginning and showed a similar rise at the end of the experiment. The potential importance of these changes in the pathomechanism of monensin poisoning is difficult to assess. Although it cannot be considered a proven fact based upon the curve, it may be supposed that the load constituted by enhanced lipid peroxidation induced an increase in liver GSH-Px activity. This, together with the observation that sheep fed nutritive doses of monensin had significantly elevated blood GSH-Px activity (Anderson et al., 1983), indicates that a relationship exists between monensin and certain variables related to lipid peroxidation. On the other hand, it is worth mentioning that in our experiment the chicks were akinetic over a rather long period during which time they did not feed. Fasting is known to substantially decrease the reduced glutathione content of the liver in the first place (Comporti, 1987). Thus, it cannot be ruled out that the drop in GSH-Px activity was due to limited substrate availability, i.e. decreased glutathione content of the liver, as glutathione is one of the substrates of GSH-Px. The elevation of GSH-Px activity, observed parallel to the improvement in the birds' clinical status, can be associated either with normalization of the glutathione level or with substrate availability ensured by radical-forming mechanisms resulting from secondary processes.

An indirect proof of the role played by lipid peroxidation processes in the development of monensin poisoning is that in calves and pigs directly or indirectly antioxidative selenium and vitamin E given parenterally prior to the

administration of highly toxic doses of monensin reduced the severity of the clinical symptoms and diminished changes in certain variables indicative of tissue injury, hereby prolonging the animals' survival (Van Vleet et al., 1983).

The toxicity of ionophore antibiotics in animals of a given species and age group may be influenced by numerous factors primarily related to feeding (Bartov and Jensen, 1980; Bartov, 1986; Parsons et al., 1984) and medication (von Dorn et al., 1983; Laczay et al., 1987). To our present knowledge, at least part of these effects may manifest itself as a positive or negative influence on hepatic lipid peroxidation status which is of prime importance in the metabolism and detoxification of ionophores. The significantly elevated catalase activity found throughout this experiment may indicate enhanced detoxifying function of the liver. The sole substrate of catalase is hydrogen peroxide (Mézes and Matkovics, 1986) which appears during lipid peroxidation and as an end-product of oxidative detoxification (Comporti, 1987).

We cannot give a satisfactory explanation for the fact that lipid peroxidation parameters were unchanged in the breast muscle throughout and that they even decreased slightly in the treated group. As the dose applied comes close to the single acute LD_{50} value of monensin (200 mg/kg body mass), it cannot account for the practically unchanged lipid peroxidation indices of muscles (breast muscle). In ionophore antibiotic poisoning of poultry, at least at a young age, the breast muscle suffers much less damage than different muscle group of the thigh. Thus, it is possible that the muscle portion examined in this study did not suffer oxidative damage. Without histopathological examination this possibility can be neither corroborated nor excluded. Intracellular calcium accumulation takes place and is known to play an important role in the pathogenesis of monensin poisoning. After inducing intracellular calcium accumulation by other methods, Belluk et al. (1988) reported enhanced lipid peroxidation processes and found that simultaneous adminstration of antioxidants failed to prevent damage of the muscle tissue. This seems to confirm our original supposition that lipid peroxidation processes play a role in the pathogenesis of muscular damage. However, further studies are needed to shed light on these matters at issue.

To sum up the findings of this work: overdosage of monensin resulted in marked enhancement of lipid peroxidation processes in the liver, while in the given experimental design the lipid peroxide status of the blood plasma and breast muscle remained practically unchanged.

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TOXICOLOGICAL AND BIOLOGICAL STUDIES ON JAPANESE QUAILS FED GRADED LEVELS OF FURAZOLIDONE

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Furazolidone (FZ) was administered to 42-day-old female Japanese quails as a feed additive at doses of 0, 200, 400, 600 and 800 ppm for a period of 28 days. Dose-dependent effects were observed. High levels of FZ (600 and 800 ppm) significantly altered growth, decreased feed consumption, caused marked atrophy of the ovaries and oviducts leading to cessation of egg laying, and resulted in higher mortality. Hepatotoxicity was evidenced by an increase in serum aspartate aminotransferase (AST), alanine aminotransferase (ALT) and alkaline phosphatase and a decrease in serum total protein, in addition to degenerative changes of the hepatocytes in FZ-treated birds. A rise in serum urea was also observed. Symptoms leading to death included a loss of appetite causing emaciation followed by nervous disturbances (compulsive movements and circling). No signs of cardiomyopathy were observed. Japanese quails did not tolerate FZ at a concentration (400 ppm) recommended for the prevention of salmonellosis in poultry.

Keywords: Furazolidone, Japanese quail, toxicological and biological study

Although there has been a growing interest in the intensive production of Japanese quail *(Coturnix coturnix japonica)*, little is known about the efficacy and safety of antibacterial drugs in this avian species. In this work we evaluate safety of furazolidone (FZ), an antibacterial drug widely used as a feed additive in poultry for the prevention and treatment of salmonellosis (Bywater, 1982) which has been reported to cause natural outbreaks in the quail (Pomeroy, 1979). Field cases of FZ toxicity have been reported to occur as a result of mixing errors (Sályi et al., 1986) and in flocks of ducklings given therapeutic concentrations of the drug (Reed et al., 1987). The heart was the major organ involved in FZ toxicity in the avian species investigated (chicken, turkeys and ducks). If added to the feed at levels of 700 ppm or more, FZ was found to produce cardiomyopathy, cardiomegaly and ascites (Czarnecki, 1980; Ali and Bartlet, 1982; Czarnecki et al., 1983; Van Vleet and Ferrans, 1983*b*). Cardiomyopathy induced by FZ is very similar to that in round heart disease, a spontaneously occurring cardiomyopathy affecting turkeys and chicken (Czarnecki et al., 1975). Thus, FZ-induced cardiomyopathy in turkeys and ducks has been used as a model to investigate other drug-induced cardiomyopathies,

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especially those in which symptoms of congestive heart failure occur (Czarnecki, 1980; Czarnecki and Evanson, 1980; Czarnecki et al., 1983; Powers et al., 1983; Van Vleet and Ferrans, 1983*a*, *b* and *c*).

To our knowledge, no reports are available about the response of Japanese quails to FZ. So, the present study was carried out to investigate some biological and toxicological effects of various levels of FZ on Japanese quails to determine the suitable safety level, if any, to be applied to this species. Moreover, if proved to be susceptible to FZ cardiomyopathy, the quail would provide a much cheaper disease model.

Materials and methods

Drug

Furazolidone (FZ), a veterinary preparation produced by VETWIC (Veterinary Division, El-Nasr Pharmaceutical Chemicals Co., Egypt) was administered orally as a feed additive in graded levels from 200 to 800 ppm (200–800 mg/kg).

Birds

One hundred 42-day-old female Japanese quails with an average body mass of 135.3 $\pm$ 1.79 g ($\bar{x} \pm$ SEM) were taken at random from the flock bred at the Poultry Research Farm of Kafr-El-Sheikh, Faculty of Agriculture, Tanta University, Egypt. Birds were divided into five experimental groups of 20 quails each and were kept by group in metal batteries where fresh water and adequate diet (according to NRC, 1984) were provided ad libitum.

Experimental design

The five experimental groups were randomly assigned to one of the following treatments:

Group I: fed a diet containing 200 ppm FZ;
Group II: fed a diet containing 400 ppm FZ;
Group III: fed a diet containing 600 ppm FZ;
Group IV: fed a diet containing 800 ppm FZ;
Group V: served as control and fed the basal diet without any additives.

The experiment lasted for 28 days. The birds were weighed individually at 0, 7, 14, 21 and 28 days of treatment. Group feed consumption data were recorded at the same intervals. Group egg production was recorded daily and egg production was calculated on a hen per day basis. Birds were frequently observed to detect any clinical alterations that might occur and those that died during the experiment were subjected to postmortem examination.

Histopathological and biochemical studies

These were performed twice throughout the experiment. On the 14th and 28th day of FZ administration five birds were taken randomly from each group, slaughtered, the blood was collected, centrifuged and the serum was frozen and kept for later analyses. The liver, ovary and heart of each bird were morphologically examined for any abnormalities, then carefully removed, weighed and kept in 10% formal saline. Thin sections (6 microns) were prepared from livers and ovaries and stained with haematoxylin and eosin as described by Humason (1979). Cross-sections were taken at different levels in the heart venticular lumina and examined for dilatation.

Serum samples were analysed for the activities of alkaline phosphatase, aspartate aminotransferase (AST), alanine aminotransferase (ALT), for total protein content and blood urea concentration using commercial diagnostic kits (Bio Merieux, France).

Statistical analysis

Data obtained were statistically analysed according to Snedecor and Cochran (1973).

Results

Effect of furazolidone (FZ) on growth, feed consumption and mortality

Average live body mass was significantly reduced by including FZ in the diet at doses higher than 400 ppm (Table I). After 14 days of administration birds of the control (group V) were significantly heavier ($P < 0.05$) than those of groups receiving 600 or 800 ppm of the drug (groups III and IV), but slightly (nonsignificantly) heavier than dose of groups receiving 200 or 400 ppm FZ (groupsI and II, $P < 0.05$). Body mass gains in proportion to initial body mass were 24.3 %,12.1%, —3.1%, —18.9% and 28.8% for groups I, II, III, IV and V, respectively. By the end of the experimental period (28days) the final body mass of the four treated groups (I, II, III, IV) was about 95.5%, 92.1%, 83.2% and 70.3% of that of the control group, respectively.

Increasing the dose of FZ in the diet up to 800 ppm markedly reduced the amount of food consumed especially during the first two weeks of administration (Table I).

Effect of FZ on rate of laying expressed as the percentage of egg production on a hen/day basis is given in Table II. Adding FZ at doses over 200 ppm significantly depressed egg production ($P< 0.05$). The effect became more expressed as the dose increased. 800 ppm FZ in rations of quails caused complete cessation of egg laying within a few days.

Table I

Biweekly records of live body mass ($\bar{x} \pm$ SEM) and amount of feed consumed (g/bird/day) as influenced by furazolidone administration

Experimental groups	Traits	Duration of furazolidone administration (weeks)		
		0	2	4
Group I	Body mass (g)	135.6±3.68	168.5±4.01^{a*}	181.9±2.79^{ab}
(200 ppm FZ)	Feed consumption		21.8	25.2
Group II	Body mass (g)	139.5±3.35	156.4±3.20^{a}	175.4±4.08^{b}
(400 ppm FZ)	Feed consumption		22.3	24.7
Group III	Body mass (g)	141.4±4.13	137.0±6.78^{b}	158.5±7.51^{c}
(600 ppm FZ)	Feed consumption		20.3	23.6
Group IV	Body mass (g)	132.7±4.49	107.6±6.41^{c}	134.0±7.84^{d}
(800 ppm FZ)	Feed consumption		13.8	20.9
Group V	Body mass (g)	132.5±4.01	170.7±4.59^{a}	190.5±2.93^{a}
(control)	Feed consumption	24.4		26.7

* Means within column having different alphabetical superscripts are significantly different at $P \leq 0.05$

Table II

Weekly mortality records and rate of laying as influenced by furazolidone administration

Experimental groups	Traits	Duration of furazolidone administration			
		1	2	3	4
Group I	Deaths	—	—	—	—
(200 ppm FZ)	Rate of laying	55.7^{a*}	60.1^{a}	60.8^{a}	63.5^{a}
Group II	Deaths	—	—	—	—
(400 ppm FZ)	Rate of laying	30.5^{b}	28.8^{b}	31.1^{b}	29.2^{b}
Group III	Deaths	—	2	2	1
(600 ppm FZ)	Rate of laying	10.2^{c}	6.7	2.2	2.6^{c}
Group IV	Deaths	2	5	3	—
(800 ppm FZ)	Rate of laying	4.3	0.0^{c}	0.0^{c}	0.0^{c}
Group V	Deaths	—	—	—	—
(control)	Rate of laying	58.2^{a}	62.3^{a}	62.5^{a}	63.3^{a}

Rate of laying is expressed as the percentage of egg production calculated on a hen/day basis; *Means within columns having different alphabetical superscripts are significantly different at $P \leq 0.05$

Deaths occurred only in groups receiving FZ at doses of 600 ppm (group III) and 800 ppm (group IV). By the 14th day of administration the mortality rate was 10% and 35% for the two groups, respectively. Total mortality rate for the whole experiment reached 20% for group III and 50% for group IV. Symptoms leading to death were loss of appetite leading to emaciation followed by nervous disturbances (compulsive movements and circling). Signs of severe

starvation (empty and markedly atrophied alimentary canal) were observed in most cases that occurred in the first two weeks. Examination of quails that died naturally and of experimentally slaughtered birds did not reveal any signs of ascites, oedema, round heart or hydropericardium. The surface of the liver and heart was smooth and glistening.

Effect of furazolidone (FZ) on heart, liver and ovary

The results presented in Table III clearly show that FZ given at doses up to 800 ppm did not affect relative heart weight. Differences observed in the absolute values (mg) are due to the significant variations in live body mass. When dissected, the heart appeared normal without any signs of cardiomegaly or cardiomyopathy (Fig. 1*b* and *c*).

Liver weight, expressed either in mg or as a percentage of live body mass was significantly ($P < 0.05$) decreased by FZ treatment. By the 14th day of

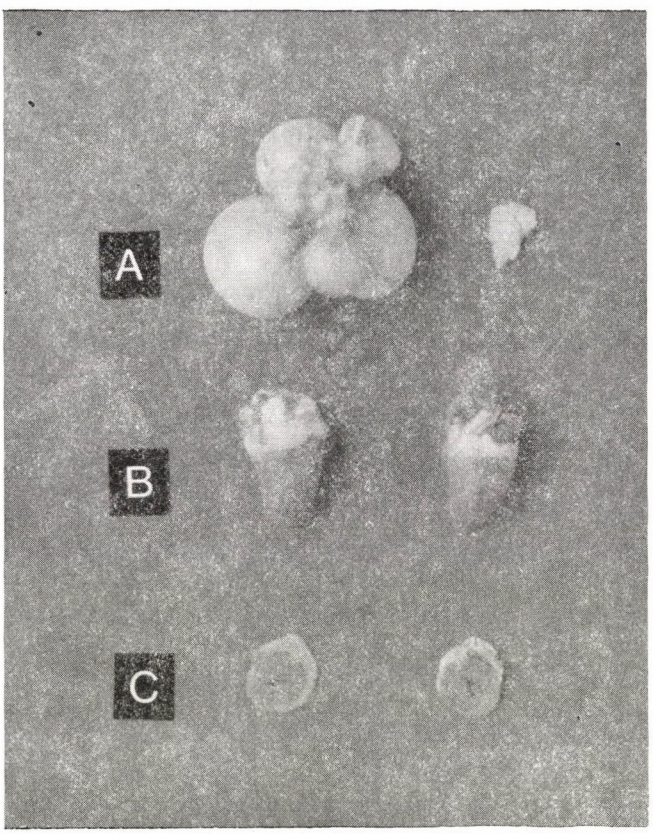

Fig. 1. The ovary and heart of quails fed a diet containing 800 ppm furazolidone (right) as compared to those of control birds (left)

Table III

Mass of heart, liver and ovary (mean±SEM) as influenced by furazolidone (FZ) administration+

Experimental groups	Live body mass (g)	Heart		Liver		Ovary	
		(mg)	(%)*	(mg)	(%)	(mg)	(%)
		Average values after 14 days of administration					
Group I (200 ppm FZ)	157.2 ± 7.57	1728±[b]* 101	1.08[a]	4141±[b] 402	2.62[ab]	2545±[ab] 1284	1.54[a]
Group II (400 ppm FZ)	150.6 ± 6.19	1400±[bc] 84	0.94[a]	3772±[b] 135	2.51[b]	339±[bc] 69	0.22[b]
Group III (600 ppm FZ)	107.4 ± 9.04	1034±[cd] 55	0.98[a]	2530±[c] 238	2.35[b]	108±[c] 16	0.10[b]
Group IV (800 ppm FZ)	82.2 ± 8.65	828±[d] 43	1.01[a]	1847±[c] 175	2.23[b]	104±[c] 16	0.13[b]
Group V (control)	176.4 ± 5.52	1968±[a] 259	1.11[a]	5303±[a] 272	3.02[a]	3310±[a] 1257	1.80[a]
		Average values after 28 days of administration					
Group I	180.6 ± 6.00	1346±[b] 49	0.80[a]	5850±[a] 369	3.23[a]	5017±[a] 714	2.74[a]
Group II	159.4 ± 12.04	1421±[b] 154	0.88[a]	3492±[b] 312	2.20[b]	1098±[b] 450	0.64[b]
Group III	154.0 ± 11.33	1414±[b] 144	0.91[a]	3780±[b] 609	2.39[b]	780±[b] 478	0.46[b]
Group IV	133.8 ± 8.87	1233±[b] 172	0.90[a]	2834±[b] 288	2.10[b]	55±[b] 9	0.04[c]
Group V	200.4 ± 4.43	1703±[a] 135	0.85	7435±[a] 538	3.74[a]	4424±[a] 466	2.20[a]

+n = 5; * % calculated in proportion to live body mass; *For each period, means within column having different alphabetical superscripts are significantly different at $P \leq 0.05$

administration average liver weight (in mg) for birds receiving 800 ppm comprised only about 35% of that for control birds and by the end of the experiment it did not exceed 38%. If adjusted to live body mass the two ratios reached 74% and 56%, respectively. Histopathological examination of livers from FZ-treated birds (receiving 800 ppm) showed severe degenerative changes.

Ovaries of birds given diets containing 600 ppm or 800 ppm FZ were drastically atrophied by the second week of treatment and appeared as if birds were immature (Fig. 1*a*), being compact, triangular, flattened with small follicles giving the surface a granular appearance. Average ovarian weight for those two groups was only about 3.27% and 3.14%, respectively, of that for the control group. Histological examination of ovarian sections confirmed the results of organ weights. The ovarian cortex of FZ-treated birds (800 ppm)

Table IV

Effect of furazolidone administration on serum alkaline phosphatase (AP), aspartate aminotransferase (AST), alanine aminotransferase (ALT) activity, and total protein (TP) and urea concentration ($\bar{x} \pm$ SEM)

Experimental groups+	AP (K.A/dl)	AST (IU/dl)	ALT (IU/dl)	TP (g/dl)	Urea (mg/l)
	Average values after 14 days of administration				
Group I (200 ppm FZ)	36.7 $\pm^{d*}$ 2.55	119.0 $\pm^{d}$ 2.06	15.3 $\pm^{cd}$ 0.68	5.0 $\pm^{a}$ 0.39	8.8 $\pm^{d}$ 0.35
Group II (400 ppm FZ)	42.6 $\pm^{cd}$ 2.11	138.4 $\pm^{ab}$ 7.22	15.7 $\pm^{cd}$ 1.51	4.4 $\pm^{ab}$ 0.14	10.6 $\pm^{bcd}$ 0.77
Group III (600 ppm FZ)	46.6 $\pm^{bc}$ 2.32	148.6 $\pm^{a}$ 3.76	24.2 $\pm^{ab}$ 1.0	3.2 $\pm^{cd}$ 0.24	13.6 $\pm^{bc}$ 1.47
Group IV (800 ppm FZ)	50.3 $\pm^{ab}$ 2.48	148.0 $\pm^{a}$ 2.73	26.7 $\pm^{a}$ 1.21	3.9 $\pm^{b}$ 0.34	15.5 $\pm^{b}$ 1.27
Group V (control)	37.4 $\pm^{d}$ 2.44	121.5 $\pm^{cd}$ 2.05	12.9 $\pm^{b}$ 0.42	5.1 $\pm^{a}$ 0.42	7.6 $\pm^{d}$ 0.83
	Average values after 28 days of administration				
Group I	38.4 $\pm^{d}$ 2.12	111.6 $\pm^{d}$ 5.29	13.4 $\pm^{cd}$ 0.55	3.8 $\pm^{bc}$ 0.24	9.2 $\pm^{cd}$ 1.10
Group II	47.6 $\pm^{bc}$ 2.45	130.1 $\pm^{bc}$ 1.53	16.1 $\pm^{c}$ 0.96	3.6 $\pm^{bcd}$ 0.19	11.3 $\pm^{bcd}$ 2.24
Group III	49.3 $\pm^{b}$ 2.39	138.4 $\pm^{ab}$ 3.83	22.2 $\pm^{b}$ 1.59	3.3 $\pm^{cd}$ 0.23	13.6 $\pm^{bc}$ 2.59
Group IV	55.8 $\pm^{a}$ 2.61	147.7 $\pm^{a}$ 1.73	27.5 $\pm^{a}$ 1.08	2.9 $\pm^{d}$ 0.24	28.5 $\pm^{a}$ 4.15
Group V	40.6 $\pm^{b}$ 2.19	112.3 $\pm^{d}$ 2.06	13.3 $\pm^{cd}$ 0.30	4.6 $\pm^{a}$ 0.25	7.9 $\pm^{d}$ 0.46

+ n = 5; * Means within column having different alphabetical superscripts are significantly different at $P \leq 0.05$

consisted of a mass of immature follicles with a less vascularized medulla. Also oviductal length and diameter were markedly reduced in the treated birds.

Effect of furazolidone (FZ) on serum alkaline phosphatase, AST, ALT, total protein and urea

Biochemical blood analyses (Table IV) clearly showed that FZ administration significantly inhibited liver functional activity. Serum alkaline phosphatase, AST and ALT were significantly increased ($P < 0.05$) by FZ administration in a dose-dependent manner. This was accompanied by a parallel decrease in serum total protein concentration. At the same time, blood urea concentration was significantly elevated, suggesting that also the kidneys may be affected by high levels of the drug.

Discussion

Various avian species show interesting differences in their susceptibility to FZ cardiomyopathy. Previous investigations showed that FZ possesses a selective myocardiotoxic action in turkeys and ducks consistently causing cardiac enlargement, ventricular dilatation, hydropericardium and structural and biochemical changes in the myocardium (Czarnecki, 1980; Czarnecki and Evanson, 1980; Czarnecki et al., 1983; Powers et al., 1983; Van Vleet and Ferrans, 1983*a*, *b*, *c*). In chickens, FZ inconsistently caused cardiomyopathy (Jensen et al., 1975; Sályi et al., 1986). In the present study, FZ given at a dose of 200–800 ppm failed to increase heart weight or cause ventricular dilatation or any histological changes in the myocardium. These findings show that the quail cannot be used as a disease model for FZ-cardiomyopathy.

The doses related to a decrease in feed consumption and body mass observed in this study are consistent with previous reports on the growth-retarding effect of FZ in chickens and turkeys (Jensen et al., 1975) and in ducklings (Van Vleet and Ferrans, 1983*c*).

Dose-dependent atrophy of the ovaries and oviducts leading to a sharp decrease in egg production was observed in FZ-fed birds. This agrees with the results obtained by Hernandez and Martinnez (1984) and Quesada et al. (1986) who reported that FZ exerts adverse effects on testis morphology and semen characteristics. Thus, FZ seems to have an inhibitory effect on the reproductive system in both male and female birds. Therefore, it is preferable to avoid applying it in breeding flocks, especially when high doses (400 ppm) are needed.

FZ was found to have hepatotoxic effect in the quail as it caused a decrease in liver weight (Table III), degenerative changes in hepatocytes, an elevation in AST, ALT, alkaline phosphatase activities and a decrease in serum total protein concentration (Table IV). The progressive fall in the concentration of serum protein is in agreement with the results obtained by Khomenko and Khomenko (1987) who showed that FZ causes a decrease in blood and liver amino acid content. The observed rise in urea concentration (Table IV) may be attributed to a nephrotoxic effect and/or an excessive breakdown of tissues.

Toxicological effects of FZ have been thought to be caused by inhibition of monoamine oxidase (Ali and Bertlet, 1982) or failure of energy metabolism by inhibition of glycogenolysis (Czarnecki and Evanson, 1980). Recent evidence indicates that FZ is metabolized into a chemically reactive intermediate which becomes covalently bound to cellular macromolecules (Vroomen et al., 1987). It is known that reactive metabolites can mediate a wide variety of toxicological effects (Gillette et al., 1974) such as hepatotoxicity observed in this study.

The results of this study show that the Japanese quail did not tolerate the concentration of FZ recommended for the prevention of salmonellosis (400 ppm)

in poultry (Bywater, 1982). When its use cannot be avoided, it would not be enough to choose the right dose: good mixing would also have to be ensured to avoid serious toxicological effects and mortality.

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RESTRICTION SITE MAPPING OF BOVINE ADENOVIRUS TYPE 1

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Physical maps of the DNA of bovine adenovirus (BAV) type 1 were established using *Apa*I, *Bam*HI, *Bst*EII, *Eco*RI and *Kpn*I enzymes. The size of the viral genome was found to be around 35,000 base pairs. The orientation of the maps was determined by hybridizing Southern blots of BAV-1 DNA with the cloned hexon gene region of BAV-3.

Keywords: Bovine adenovirus, DNA, restriction enzymes, physical map

Bovine adenoviruses causing usually mild respiratory and/or enteric diseases in calves are widely distributed all over the world (Bürki, 1990). With nine presently accepted serotypes, the group of BAVs is, right after the human and simian adenoviruses, the third most numerous group of Mastadenoviruses (for review see Mautner, 1989). The possible evolutionary pathway and the relationship between adenoviruses of different host origin is a puzzling question of today's virus taxonomy. Besides simian (Wigand et al., 1989), murine (Ball et al., 1989) and canine (Shibata et al., 1989; Spibey et al., 1989) adenoviruses, BAVs have also been included into DNA comparative studies (Takács et al., 1983; Belák et al., 1983; Belák et al., 1986; Hu et al., 1984*a* and *b;* Toogood and Hay, 1988; Benkő et al., 1990).

According to the suggestion of Bartha (1969), BAVs are divided into two subgroups. Subgroup 1 contains serotypes 1, 2, 3 and 9, while subgroup 2 comprehends serotypes 4, 5, 6, 7 and 8. DNA restriction enzyme (RE) analysis of the reference strains did not reveal obvious genetic relatedness between the different bovine, or between the bovine and human types (Benkő et al., 1988). By DNA hybridization experiments (Benkő et al., 1990), however, a very close genetic relationship was demonstrated among the subgroup 2 BAVs, while no cross-reaction could be observed between subgroup 1 and subgroup 2 BAVs, or between subgroup 2 BAVs and human adenoviruses (HAVs). The DNA homology detectable between the examined HAVs and the subgroup 1 BAVs was variable, but in general rather weak. Correct localization of the hybridizing sequences was not possible in the case of BAV-1, as no physical maps were available. Restriction site maps of BAV-1 were also needed for genetic comparisons among subgroup 1 BAVs.

In this work physical maps of BAV-1 constructed by the use of five REs are described.

Materials and methods

The prototype strain of BAV-1 designated No. 10 (Klein et al., 1959) was propagated on tertiary calf kidney cells. The virus concentration and purification methods as well as the techniques of DNA extraction and agarose gel electrophoresis were described earlier (Benkő et al., 1988). The viral DNA was digested with REs purchased from Amersham according to the standard protocols of the manufacturer. The size of the DNA fragments generated by *Apa*I, *Bam*HI, *Bst*EII, *Eco*RI, and *Kpn*I enzymes was determined graphically using agarose gels of different concentrations (0.4–1.8%) and different molecule mass standards (*Bst*EII or *Hin*dIII cut λ phage DNA) depending upon the size of the fragments to be measured.

Southern blotting and DNA hybridization were carried out basically as described by Maniatis et al. (1982). Hybridization was done without formamide and under "less stringent" conditions, i.e. annealing at 55 °C overnight and washing at 55 °C in 6×SSC and 0.1% SDS. The cloned *Eco*RI/C fragment (covering the hexon gene region) of BAV-3 (Harrach, unpublished results) was used as hybridization probe. The probe labelling was done using an Amersham random priming kit with ^{32}P containing dCTP.

Results

The five applied REs, *Apa*I, *Bam*HI, *Bst*EII, *Eco*RI and *Kpn*I had, respectively, 1, 6, 2, 7 and 1 recognition sites on the genome of BAV-1 (Figs 1*a* and 2). The estimated size of the DNA fragments generated by each enzyme is shown in Table I. Similarly, the size of the fragments resulting from double or

Table I

The size of DNA fragments generated by the cleavage of the BAV-1 genome with different REs

	*Apa*I	*Bam*HI	*Bst*EII	*Eco*RI	*Kpn*I
A	18,250	13,060	20,190	11,990	18,630
B	16,750	6,600	12,910	6,860	16,370
C		4,950	1,900	4,740	
D		3,710		4,230	
E		3,250		2,830	
F		2,550		2,600	
G		800		1,030	
H				720	
Total	35,000	35,000	35,000	35,000	35,000

The size is given in base pairs (bp)

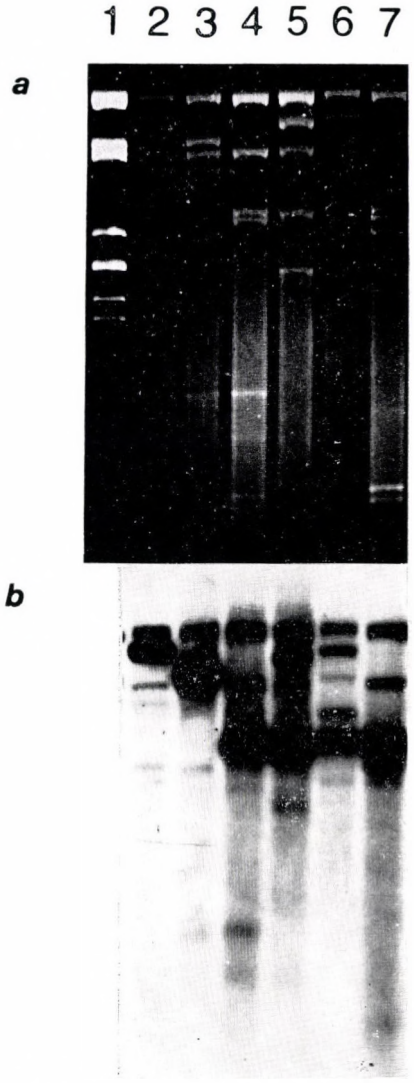

Fig. 1. BAV-1 DNA resolved by electrophoresis through 1% agarose gel. (*a*): Lane 1: *Pst*I digest of λ phage DNA; lanes 2 to 7: BAV-1 DNA digested with *Eco*RI (2), *Eco*RI + *Kpn*I (3), *Bam*HI + *Eco*RI + *Kpn*I (4), *Bam*HI + *Kpn*I (5), *Bam*HI (6) and *Bam*HI + *Eco*RI (7). (*b*) Autoradiogram of the same gel after being blotted and hybridized with the *Eco*RI/C fragment of BAV-3 DNA

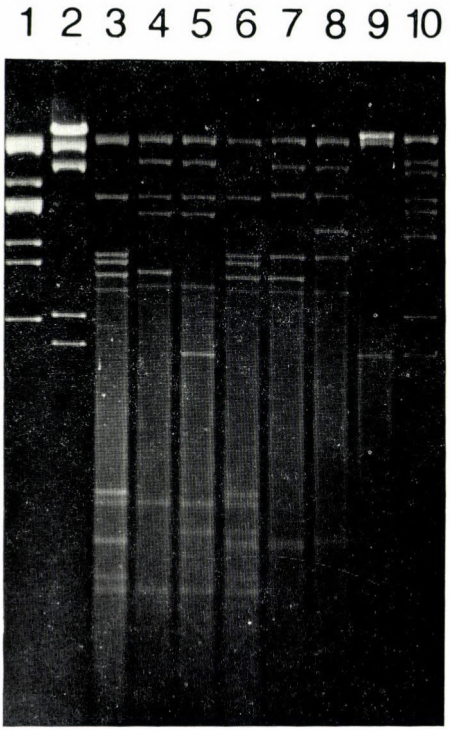

Fig. 2. BAV-1 DNA resolved by electrophoresis through 1% agarose gel. (*a*): Lanes 1, 2 and 10: λ phage DNA digested with *Hpa*I, *Hind*III and *Bst*EII respectively. Lanes 3 to 9: BAV-1 DNA digested with *Bam*HI + *Eco*RI (3), *Eco*RI (4), *Eco*RI + *Bst*EII(5), *Bam*HI + *Eco*RI + *Bst*EII (6), *Bam*HI + *Bst*EII (7), *Bam*HI (8), *Bst*EII (9)

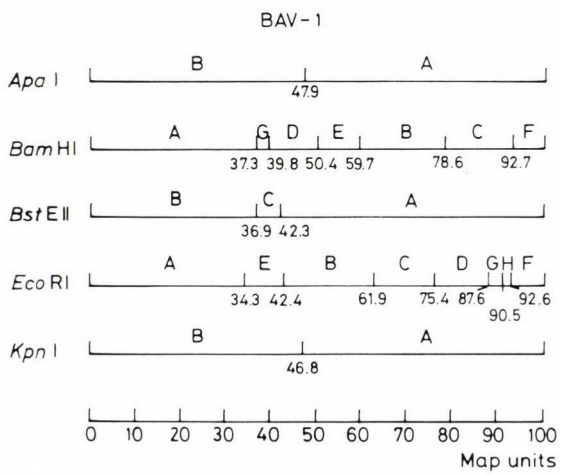

Fig. 3. Restriction site maps of BAV-1 DNA. The location of the particular restriction sites is given in map units. The maps are divided into 100 units and 1 map unit is equal to approximately 350 bp

triple digestions was also estimated, and the location of the particular cleavage sites on the BAV-1 genome was determined by overlapping these fragments.

A Southern blot made from the gel shown in Fig. 1*a* was hybridized with the cloned *Eco*RI/C fragment of BAV-3. The autoradiogram is presented in Fig. 1*b*.

The physical maps of BAV-1 DNA are shown in Fig. 3.

Discussion

Subgroup 1 BAVs including BAV-1 have been found to be related to HAVs and other Mastadenoviruses in their biological and serological properties (Wigand et al., 1982). DNA hybridization experiments revealed, however, that the extent of the genetic relatedness of the different subgroup 1 BAV types to HAV-2 was variable, and very low in the case of BAV-2 (Belák et al., 1983) and BAV-1 (Benkő et al., 1990). In the work presented in this paper, physical maps of the BAV-1 genome were constructed with several REs to facilitate a more correct genetic comparison and gene mapping of BAV-1.

DNA fragments resulting from double or triple RE digests were overlapped by their sizes through the single digested fragments. The genome size of BAV-1 calculated from the size of the specific restriction fragments was 35,000 bp, and this value is similar to that found for BAV-3. We supposed, therefore, that the gene organization of the two BAV genomes is also similar. The result of the Southern blot hybridization gave a good starting point in orienting the maps. The cloned *Eco*RI/C fragment of BAV-3 representing a part of the genome (including the hexon gene region) from map unit 44.7 to 63.3 was used as hybridization probe. With the less stringent hybridization conditions, all fragments became visible, but unambiguous positive hybridization occurred with the *Eco*RI/B and E and with the *Bam*HI/D and E fragments. The maps were constructed supposing that these fragments correspond approximately to the same region (i.e. between map units 44 and 64) of the BAV-1 genome.

The presence of deletions up to 13% of the total length at the right end of the DNA has been reported for BAV-3 by Kurokawa et al. (1978). The BAV-1 virions applied for this study had not been purified through CsCl gradient, thus the DNA was extracted from a mixture of both the complete and incomplete virions. Accordingly, as it can be seen in Figs 1 and 2, the occurrence of DNA fragments present in submolar quantities was observed. Besides the complete *Bam*HI/F and *Eco*RI/F fragments, at least two additional minor populations of deleted (by approximately 100 and 200 bp) DNA fragments were detected.

Further experiments, including additional mapping and molecular cloning of BAV-1 are planned to facilitate gene mapping and detailed genetic and comparative studies of this virus.

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ALTERNATING ONE-LETTER SYMBOLS FOR THE REPRESENTATION OF CODON VARIATIONS AT THE LEVEL OF AMINO ACID SEQUENCES

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An idea is presented to use different letter types (e. g. L. 1, *L*, *1*, ^{L}, $_{l}$) for the alternating triplets coding the same amino acids. Such compact and demonstrative amino acid sequence comparisons can make easy the identification of differences at the DNA level if not seen in amino acid sequences (e. g. for choosing specific or non-specific primers for polymerase chain reaction).

Keywords: DNA sequences, amino acid sequences, sequence homology, PCR

Comparing amino acid sequences of related genes to study their homology and to get hints about their evolutionary distance is a widely used and very demonstrative method (Albrecht and Fleckenstein, 1990; Fitzpatrick et al., 1989; Honess et al., 1989). However, the occurrence of amino acids (possible mutations) at certain points is under selective pressure because of the importance of hydrophobicity values (signal sequence, transmembrane and cytoplasmic domains, etc.), the presence of special amino acids, like Cys residues (disulphide links) or motifs, like Ala-X-Thr/Ser, where X is not Pro (*N*-linked glycosylation site), Ala-X-Ala-turn (signal peptidase cleavage site), etc. to result in the right secondary and tertiary structure (enzyme activity or a form to fit into a complex structure like a viral envelope). There is a bigger freedom on the DNA level as long as the amino acids do not change (or at least not to ones causing fatal structural change). Thus DNA sequences give more information on differences between two genes and a more exact prediction of the degree of relationship.

Showing both the easily comparable one-letter-symbol amino acid sequence and the three times longer DNA sequence above that needs 6 times more space intolerable in today's lengthy sequence comparisons (Albrecht and Fleckenstein, 1990; Fitzpatrick et al., 1989; Honess et al., 1989). As a solution, we suggest applying different letter types which refer to the coding triplets, e.g. as shown in Fig. 1. (When a typewriter is used, one can apply underlining instead of italics.) In this way, one can "overlay" further information on the

A	GCT	a	GCC	*A*	GCA	*a*	GCG				Ala
C	TGT	c	TGC								Cys
D	GAT	d	GAC								Asp
E	GAA	e	GAG								Glu
F	TTT	f	TTC								Phe
G	GGT	g	GGC	*G*	GGA	*g*	GGG				Gly
H	CAT	h	CAC								His
I	ATT	i	ATC	*I*	ATA						Ile
K	AAA	k	AAG								Lys
L	TTA	l	TTG	*L*	CTT	*l*	CTC	*L*	CTA	*l*	CTG	Leu
M	ATG										Met
N	AAT	n	AAC								Asn
P	CCT	p	CCC	*P*	CCA	*p*	CCG				Pro
Q	CAA	q	CAG								Gln
R	CGT	r	CGC	*R*	CGA	*r*	CGG	*R*	AGA	*r*	AGG	Arg
S	TCT	s	TCC	*S*	TCA	*s*	TCG	*S*	AGT	*s*	AGC	Ser
T	ACT	t	ACC	*T*	ACA	*t*	ACG					Thr
Y	TAT	y	TAC									Tyr
V	GTT	v	GTC	*V*	GTA	*v*	GTG					Val
W	TGG											Trp
—	TAA	\	TAG	/	TGA							*Stop*

Fig. 1. Letter types suggested for the different coding triplets. The last column shows the three-letter symbols

compact and demonstrative amino acid sequence, making "*in situ* homology comparisons" more exact.

Such sequence comparisons make easy e.g. the location of sequences sufficiently specific (or homologous if desired) to serve as primers in polymerase chain reaction (Saiki et al., 1985).

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ISOLATION AND CHARACTERIZATION OF NEW INFECTIOUS BRONCHITIS VIRUS VARIANTS IN HUNGARY

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Two agents not agglutinating chicken erythrocytes were isolated, one in each of two flocks, from organ samples and tracheal swabs taken from 4- to 7-week-old chicks of 8 broiler flocks experiencing respiratory signs. Virus isolation was done in embryonated SPF hen's eggs. Morphological changes of the embryos, appearing as dwarfing or curling into a spherical form, usually occurred in the 3rd or 4th passage on postinoculation (PI) days 5–9. Some embryos had swollen kidneys covered with urate. Electron microscopy of ultrathin sections of these kidneys revealed the presence of virions reminiscent of coronaviruses. Similar viral particles were seen in resuspended pellets of isolates concentrated by ultracentrifugation. Based on embryo changes, cross-neutralization tests with type-specific antisera, physicochemical tests, results obtained in cell cultures, and electron microscopic findings the two isolates were identified as infectious bronchitis virus (IBV).

By cross-neutralization tests the isolates differed from IBV reference strains M41 and H52 and can be considered distinct variants. Elucidation of their epizootiological role requires further investigations.

Keywords: Infectious bronchitis virus, isolation, characterization, new variants

Infectious bronchitis (IB) of chickens occurs wherever intensive poultry qroduction is practised. Research conducted since its first description (Schalk and Hawn, 1931) has solved many problems in both epizootiology and control. IB has, however, remained a subject of interest, partly because of the fact that the causative virus is immunobiologically heterogeneous. The purpose of the work presented here was to study this problem in Hungary by virus isolation attempts from broiler flocks experiencing respiratory signs and by characterising the isolates by laboratory methods.

The first report of the occurrence of IB in Hungary based upon serological tests was by Derzsy and Lomniczi (1966). In Hungary IBV was first isolated by Lomniczi and Stipkovits (1968) from tracheal and lung samples of chicks showing respiratory signs and of laying hens with depressed egg production. Subsequently, Csermelyi and Muncz (1986) and Csontos et al. (1987) succeeded in isolating the virus.

Authors working on IB invariably agree that isolation of IBV is not an easy task. One of the preconditions of its success is that the test material is placed into a suitable growth medium as soon as possible. Also, researchers

agree that so-called blind passages are often needed for the development of characteristic embryo changes (dwarfing, curling into a spherical form, etc.).

The trachea (Csermelyi and Muncz, 1986), the lungs and the trachea (Arnaldo et al., 1965; Lomniczi and Stipkovits, 1968; Picault et al., 1986), the lungs, trachea and kidney (Ahmad et al., 1985), the lungs, trachea and tracheal swabs (El Houdafi et al., 1986), tracheal and cloacal swabs (Cook, 1984), and the ovary, oviduct and cloacal swabs (Maiti et al., 1985) may serve as material for virus isolation. The classic method of virus isolation is inoculation of suitably treated organ homogenates or swab samples into the allantoic cavity of 9- to 11-day-old embryonating eggs. IBV has also been isolated in tracheal culture (Cook, 1984) and by intratracheal inoculation of susceptible chicks (Arnaldo et al., 1965).

The success rate of virus isolation varies widely. Afrer three passages, 5 (3 cloacal swabs and 2 ovary-oviduct samples) out of 52 samples (30 ovary-oviduct samples and 22 cloacal swabs) (19.5%) taken from 30 flocks experiencing dropped egg production yielded virus (Maiti et al., 1985). In another study, 12 IBV strains were isolated from 249 samples collected from 83 diseased birds in an infected flock: 9.6%, 3.6% and 1.2% of the lung, tracheal and kidney samples, respectively, yielded virus (Ahmad et al., 1985). The success rate of virus isolation may reach 80% (Yaddin and Chai, 1984).

The isolates can be identified on the basis of embryo changes, virion morphology (Hofstad, 1978), immunofluorescence (IF) test (Csermelyi et al., 1988), cross-neutralization with type-specific antisera (Cook, 1984), plaque reduction (Hopkins, 1978), agar gel precipitation test (Lohr, 1980), haemagglutination inhibition (King and Hopkins, 1984), and physicochemical tests (Lomniczi and Stipkovits, 1968).

Materials and methods

Organ samples

Pieces taken from the trachea, lungs, kidneys and caecal lymph follicles were homogenized (Ultraturax), clarified by centrifugation at low rpm, and to each ml of the supernatant 200 μg gentamicin was added.

Tracheal swabs

Sterile cotton swabs introduced into the trachea were washed in MEM-H solution containing 200 μg gentamicin per ml. To 2 ml volumes of both the organ homogenate and the swab washings 0.5 ml of a monospecific anti-Newcastle disease virus (NDV) immune serum was added and kept at room tem-

perature for 30 min before inoculation into embryonated hen's eggs. The haemagglutination inhibition (HI) titre of the anti-NDV immune serum was 1 : 128.

Monospecific anti-NDV immune serum

A commercially available, lyophilized serum (PHYLAXIA, Budapest) inhibiting haemagglutination by NDV was used.

Egg inoculation

Five embryonating eggs were inoculated into the allantoic cavity with each sample (0.2 ml per egg) and kept at 37 °C. Embryos that died within 24 h were discarded and the others were candled twice daily.

In the initial (1st, 2nd and 3rd) passages the allanto-amnionic fluid was collected from part of the eggs between postinoculation (PI) hour 72 and 120. In the 4th and later passages the allanto-amnionic fluid was collected between PI hour 48 and 96. Non-haemagglutinating materials were stored at —20 °C until passaged further.

The remaining were opened on day 6–9 of incubation. The allanto-amnionic fluid of embryos suspected of IBV infection (dwarfed embryos, embryos curled into a spherical form, thickened amnion closely adherent to the embryo, persistence of the mesonephros containing urates, etc.) was collected separately and stored at —20 °C. A given test material was passaged maximum 6 times.

Hemagglutianation (HA) and haemogglutination inhibition (HI) test

The HA test was carried out on slides using 20% chicken erythrocytes to detect NDV. The HI test was done on microtitre plates against 4 HA units of the agent tested, using doubling dilutions of an immune serum to NDV of known titre and 1% chicken erythrocytes.

Virus strains

The following virus strains were used: IBV strain designated H52 (the strain contained in IB vaccine Bronchovac II, PHYLAXIA, Budapest), the Beaudette strain, the virulent M41 strain (8th passage, kindly provided by the Veterinary Medical Research Institute of the Hungarian Academy of Sciences), isolate B (7th passage) an isolate SZ (7th passage).

Virus propagation

Thirty 10-day-old SPF embryonating eggs were inoculated into the allantoic cavity with 1000–3000 EID_{50} of each virus strain. Embryos that died within 24 h were discarded. From the remaining embryos the allanto-amnionic fluid was collected between PI hour 30 and 36, and stored either in liquid nitrogen or at —70 °C.

EID_{50} determination

Three to five embryonating eggs were inoculated into the allantoic cavity with 0.1 ml of each dilution. Evaluation was based on embryo changes resulting from virus growth. The titre was calculated by the method of Reed and Muench (1938).

Preparation of type-specific antisera

Three to five 5-week-old SPF cockerels were serially immunized with the viruses grown as described above. The titre (EID_{50}/0.1 ml) of the virus inocula was as follows: H52: $10^{7.5}$, M41; $10^{6.5}$, isolate B: $10^{7.5}$, isolate SZ: $10^{7.5}$. On first immunization, 2 drops of the virus suspension were dropped onto the conjunctive, 0.25 ml was inoculated intratracheally and 1 ml subcutaneously. The second immunization took place 3 weeks later when 1 ml of the virus suspensions was injected intravenously. The third immunization was carried out 2 weeks later when 1 ml inactivated, oil-adjuvated vaccine was injected subcutaneously. The birds were bled on day 10–14 after the last vaccination. Virus inactivation was done with a combination of ethylene imine and formaldehyde.

Cross-neutralization tests using IBV antisera

The beta variety of the method described by Picault et al. (1986) was employed, using the Beaudette strain. The titre was the highest dilution which still neutralized.

Physicochemical tests

The isolates were tested for resistance to heat, chloroform and trypsin (Cowen et al., 1971; Lomniczi and Stipkovits, 1968) as well as cationic stabilization and stability to pH (Plummer, 1963; Wallis and Melnick, 1962).

Light and electron microscopic examination

Organ samples were fixed in 5% glutaraldehyde solution, embedded in Durcupan ACM resin, and semithin as well as ultrathin sections were cut with a UMTP-4 ultramicrotome. The sections were examined in a TESLA BS 613 electron microscope. Photomicrographs were taken at an accelerating voltage of 50 kV.

The virus-containing allanto-amnionic fluid pool was first clarified by centrifugation at 1000 *g* for 45 min at 4 °C, then sedimented by ultracentrifugation at 30,000 *g* for 60 min at 4 °C. The obtained sediment was resuspended in HEPES-buffered slaine (HBS, 5.96 g HEPES, 8.19 g NaCl and 0.15 g $CaCl_2$, made up to 1 l with distilled water and adjusted to pH 6.5 by adding 1 N

NaOH) to give 1% of the original volume of allanto-amnionic fluid. The concentrated pellet suspension was counterstained with 1% phosphotungstic acid (pH 7.0) on formvar-coated grids of 200 mesh. The preparations were examined in a TESLA BS 613 electron microscope, at an accelerating voltage of 56 kV.

Cell culture

Chick embryo kidney (CEK) cell cultures prepared from 18- to 20-day-old SPF embryos in Greiner's plastic flasks or Linbro microtitre plates were used. The cultures were inoculated with allanto-amnionic fluid containing IBV, incubated at 37 °C and checked daily in a microscope. The cell cultures were passaged on day 3 or 4. A total of 4 passages were done. The pooled material was stored at —70 °C until used for further study.

Virus titration in cell culture

The virus titre ($TCID_{50}$/0.1 ml) was determined on the basis of the cytopathic effect (CPE) by the method of Reed and Muench (1938).

Results

Virus isolation

Material from 8 flocks of 6 farms was used for virus isolation. This material included samples taken from affected birds and organs submitted to the laboratory and tracheal swabs taken at the farms. The details and results of virus isolation are shown in Table I.

Agents causing embryo changes suggestive of IBV were isolated from 2 out of the 8 flocks examined. These changes appeared distinctly in the 3rd (isolate SZ-II) and fourth (isolate B-IV) passage (Fig. 1). Further passages were needed to check whether embryo changes considered typical occurred consistently in all inoculated embryos. These changes appeared after further two passages of B-IV and one passage of SZ-II. The isolates were designated B (6th passage) and SZ (4th passage) and used for further study.

Virus isolation was unsuccessful from samples taken from birds that were at a subacute or later stage of the disease (flocks D, SZ-I and BA).

Virus isolation from organ and tracheal swab samples taken from flock SZ-II, in which the clinical signs and necropsy findings were indicative of acute IB, succeeded in the 3rd passage.

Titration of the isolates

The results of repeated titration of isolates B and SZ in embryonated hen's eggs are shown in Table II.

Table I

Infectious bronchitis virus (IBV) isolataion attempts and their results

Farm	Age of chicks examined (weeks)	Number of chicks examined	Immunization		Organ sample	Swab sample	Passage number	Result		Gross pathological finding	Remarks
			IB	ND				organ	swab		
O	4–6	6	—	+	6	—	3	neg.	—	Serous-fibrinous airsacculitis	Agent agglutinating chicken erythrocytes isolated. Agent was inhibited by anti-NDV serum
D	4–6	6	—	+	6	—	6	neg.	—	Fibrinous pseudomembranes on the airsacs, adhesions	
B-III	5	4	+	+	4	4	3	neg.	—	Not known. Only trachea and lung samples submitted	
B-IV	5	4	—	+	4	4	4	pos.	—		
SZ-I	7	6	—	+	6	6	4	neg.	neg.	Serous airsacculitis	
SZ-II	4	26	—	+	6	20	3	pos.	pos.	Serous nasal discharge, serous exudate in the trachea	All birds affected in the chicken house at sampling
BA	4	6	+	+	6	—	4	neg.	—	Serous-fibrinous airsacculitis	
Bo	4	9	+	+	9	—	4	neg.		Not known. Only tracheal samples submitted	

Table II

Titration of isolates B and SZ

Serial number of titration	Passage number	Isolate B	Isolate SZ
1	6	—	$10^{7.1}$*
2	7	—	$10^{7.5}$
3	8	$10^{7.9}$	
4	9	$10^{7.5}$	
5	10	$10^{7.5}$	

* EID_{50}/0.1 ml

Table III

Cross-neutralization tests of isolates B and SZ in embryonated hen's eggs

Virus	Type-specific antiserum			
	B	SZ	M41	H52
B	*1 : 640**	0**	1 : 5	0
SZ	<1 : 5	≥*1 : 80*	<1 : 5	0
M41	1 : 10	0	*1 : 640*	1 : 5
H52	1 : 10	1 : 5	1 : 10	*1 : 80*

* dilution which neutralizes 100 EID_{50} virus
** even the undiluted serum fails to neutralize

Identification of isolates with type-specific antisera

Results of the ovoneutralization tests of isolates B and SZ with type-specific antisera are summarized in Table III. Isolate B was neutralized by its homologous antiserum and by antiserum to strain M41 in a dilution of 1 : 640 and 1 : 5, respectively. Antisera to SZ and H52 did not neutralize isolate B. Isolate SZ was neutralized by its homologous antiserum in a dilution of ≥ 1 : 80, by antisera to B and M41 in dilutions lower than 1 : 5, and was not neutralized by antiserum to H52. The laboratory strain M41 was neutralized by antiserum to isolate B in a dilution of 1 : 10, while it was not neutralized even by undiluted antiserum to isolate SZ. The homologous serum neutralized it in a high (1 : 640) dilution while antiserum to H52 in a dilution of 1 : 5. Strain H52 was neutralized by antisera to B and SZ in a dilution of 1 : 10 and 1 : 5, respectively. The serum raised against strain M41 neutralized strain H52 in a dilution of 1 : 10 and the homologous serum in a dilution of 1 : 80.

Physicochemical tests

The results of the physicochemical tests of isolates B and SZ are given in Table IV. The two isolates proved to be labile in the heat and chloroform sen-

Table IV

Physicochemical tests of isolates B and SZ

Physicochemical test	Viruses and titres					
	B		SZ		M41	
	treated	control	treated	control	treated	control
Heat (56 °C)	0	7.8*	0	7.1	0	6.8
pH 3	5.1	7.5	4.5	6.5	5.8	6.2
Trypsin, 0.25%	6.8	7.2	6.1	6.1	6.5	7.2
Chloroform, 20%	0	6.5	0	6.5	0	7.2
Cationic stabilization	0	6.5	0	6.5	NT	NT

* EID_{50}/0.1 ml, log 10
NT: not tested

Table V

Cytopathic effect caused by, and titration of, isolates B and SZ as well as IBV strains Beaudette, M41 and H52 in cell cultures

Passage number	Strains									
	B		SZ		Beaudette		M41		H52	
	CPE	$TCID_{50}$	CPE	$TCID_{50}$	CPE	$TCID_{50}$	CPE	$TCID_{50}$	CPE	$TCID_{50}$
1	0	0	0	0	+	NT	0	0	0	0
2	0	0	0	0	+	2.5*	0	0	0	0
3	+	NT	+	NT	—	—	+	NT	+	NT
4	+	5.5	+	4.5	—	—	+	6.5	+	6.5

0 : no cytopathic effect demonstrable
\+ : cytopathic effect
* : $TCID_{50}$/0.1 ml, log 10
NT : not tested

sitivity and cationic stabilization tests. Exposure to pH 3 reduced their titre by about 2 log. Trypsin treatment had practically no effect on the virus titre. Strain M41 used as control gave the same results in the physicochemical tests.

Cytopathic effect and titration in cell culture

The cytopathic effect (CPE) and titration in cell culture of isolates B and SZ and 3 laboratory IBV strains are presented in Table V.

The two isolates and strains M41 and H52 required three passages to exert CPE. CPE was characterized by rounding-off of the cells, appearance of droplet-shaped, sharply delineated forms and formation of so-called syncytia.

Titration of isolates B and SZ in the 4th passage revealed 1 log difference between their titres. Strains M41 and H52 had the same titre which was higher than that of the isolates. The Beaudette strain was cytopathic already in the first passage; its 2nd passage had relatively low titre.

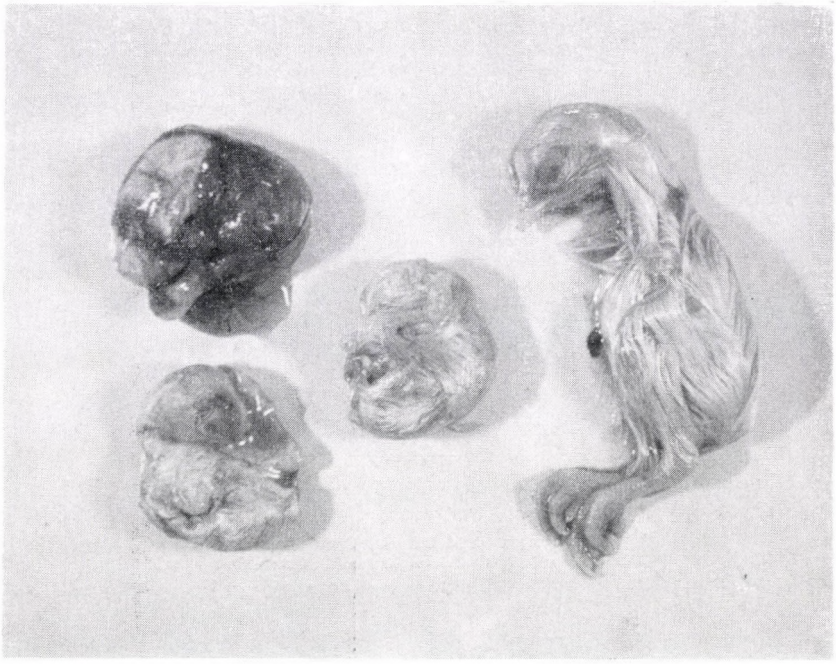

Fig. 1. Chicken embryos inoculated with isolate B (top left), isolate SZ (bottom left) and IBV strain M41 (middle) on postinoculation day 5. On the right: uninoculated control embryo

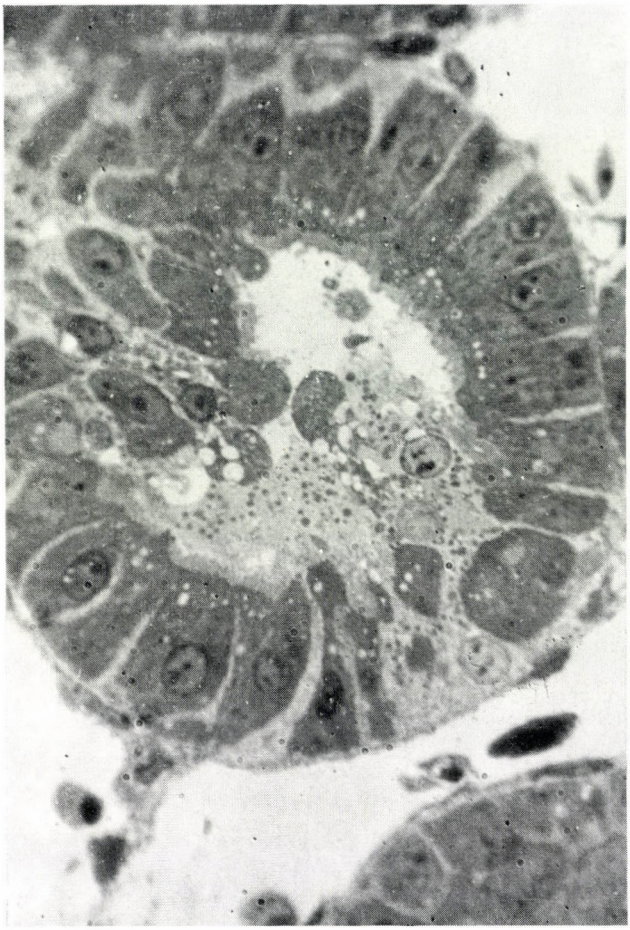

Fig. 2. Intercellular oedema and vacuolar degeneration in the kidney from an embryo inoculated with isolate B, on postinoculation day 3. ×3,400

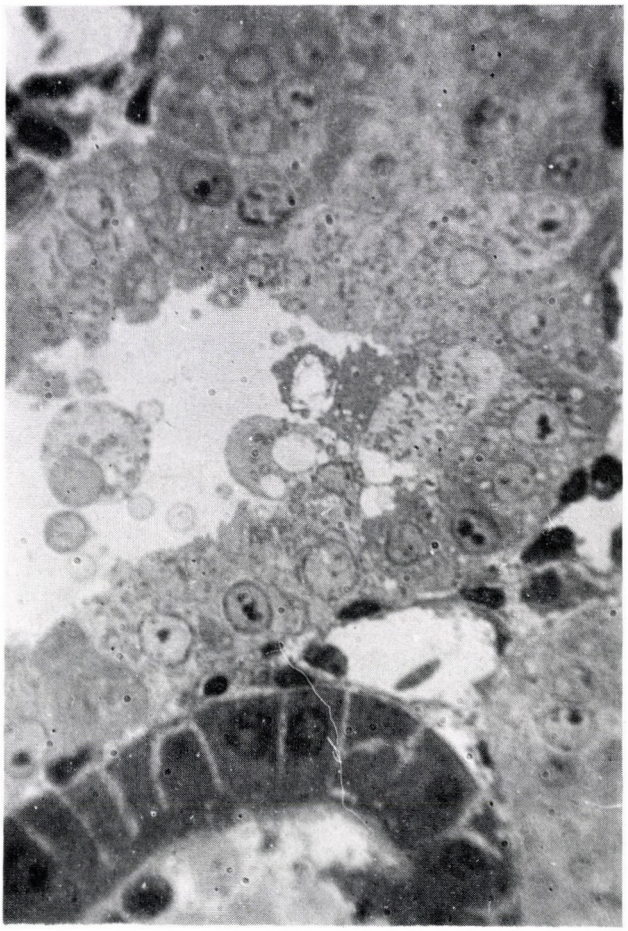

Fig. 3. Kidney from an embryo inoculated with isolate B, on postinoculation day 3. Note degeneration and necrosis in the wall of the distal tubule. ×3,400

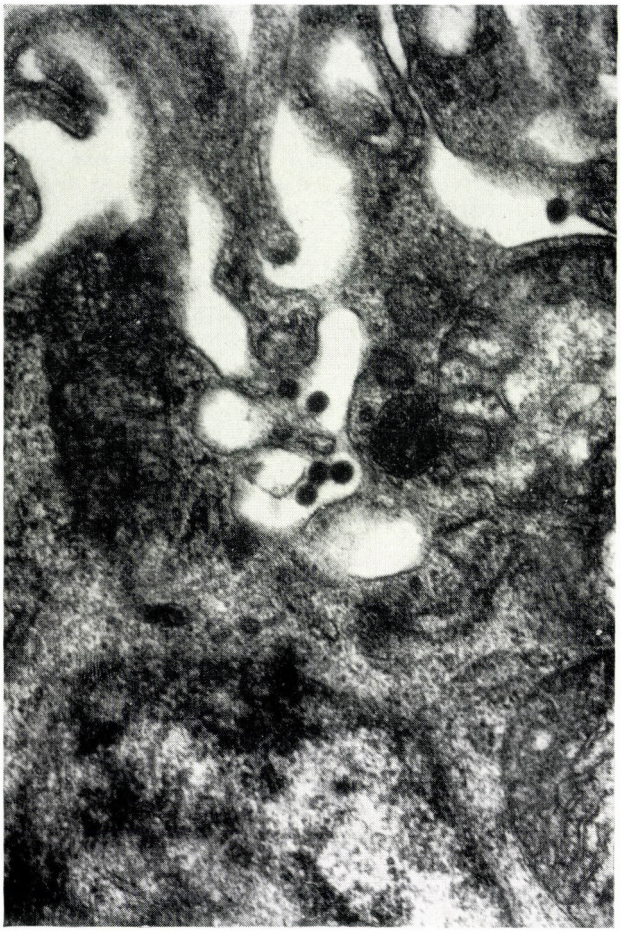

Fig. 4. Kidney from an embryo inoculated with isolate B, on postinoculation day 3. Budding virions. Electron micrograph, ×45,270

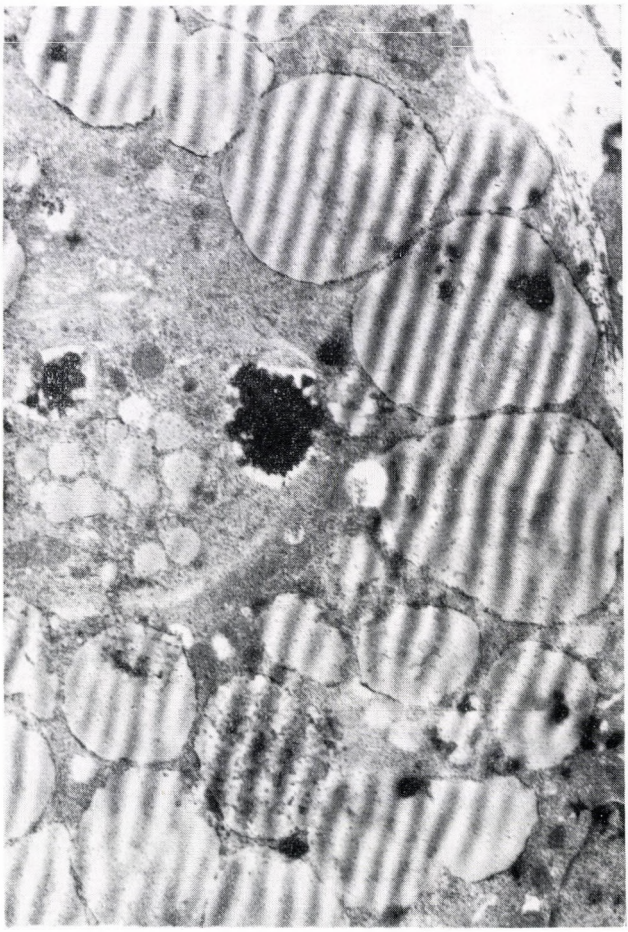

Fig. 5. Kidney from an embryo inoculated with isolate B, on postinoculation day 7. Note fatty degeneration of the tubular epithelial cells and presence of urate crystals inside and outside the tubular umen. ×7,880

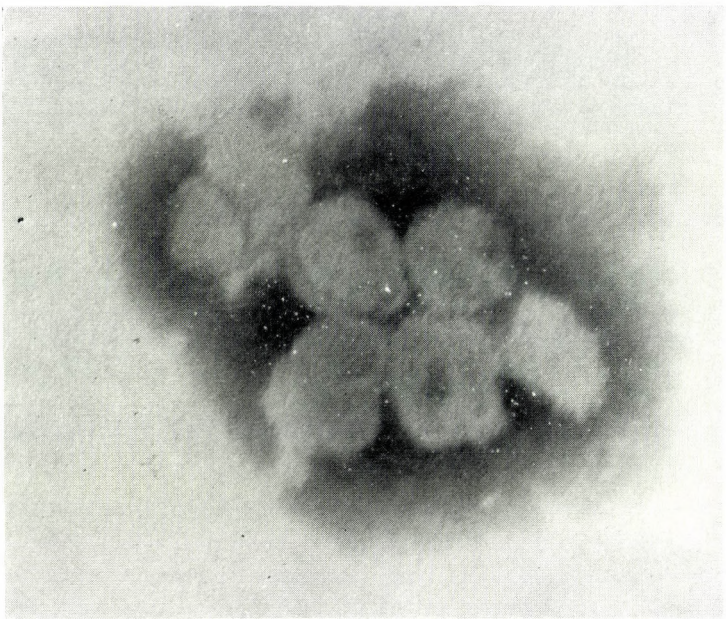

Fig. 6. Electron micrograph of the concentrated (30,000 *g*) pellet suspension of isolate B. Virions with crown-like surface projections. ×108,916

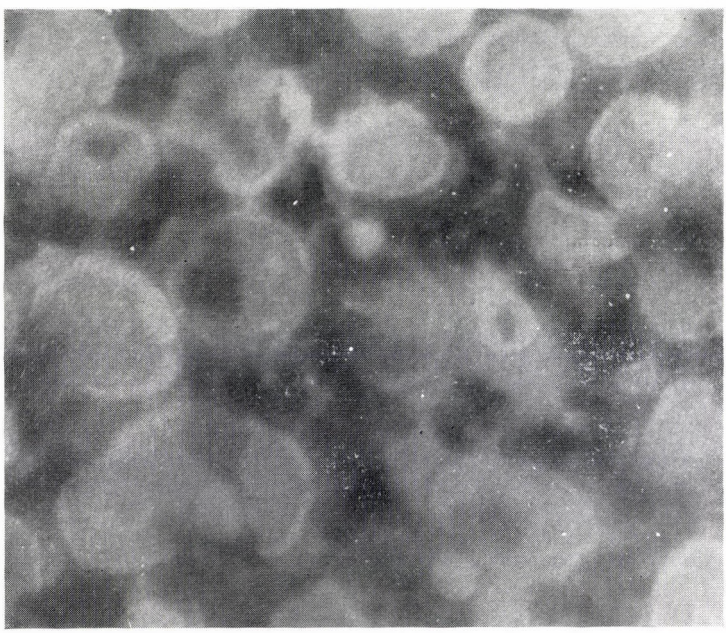

Fig. 7. Electron micrograph of the concentrated (30,000 *g*) pellet suspension of isolate SZ. Virions with crown-like surface projections. ×108,916

Embryo changes resulting from virus infection. Light and electron microscopic studies

During virus isolation, several days after inoculation the embryo becomes fixed at one place and appears as an immobile, spherical formation. In such cases very little allantoic fluid can be collected after opening the egg. The amnion is closely adherent to the embryo which "rolls out" of the egg like a ball. The unresorbed yolk sac follows the embryo as a green saccule.

In the initial (1st and 2nd) passages these changes appear in a few embryos only and develop in 5–9 days. There is no, or little, change in the majority of embryos which will hatch as healthy chicks at term. In further passages embryo changes will develop sooner and in an increasing number of embryos. In this study, 4–6 passages were needed for the appearance of typical IBV-related changes in all embryos.

Three days after inoculation the kidney of the embryos was swollen and pale. Histologically interstitial and intercellular oedema and haemorrhages were seen, together with necrosis in the epithelium of the proximal and distal tubules. Some cells showed vacuolar degeneration while others fell into the lumen of tubules like a mass of indistinct structure, in some places filling the lumen completely (Figs 2 and 3).

Electron microscopic examination of the kidney revealed the presence of spherical viral particles, 100 to 110 nm in diameter, of coronavirus morphology (Fig. 4).

One week after inoculation the embryos' kidney was swollen and pale, with a fingerprint-like pattern on its surface. The renal tubules and the ureter were filled by urate crystals and the epithelial cells showed fatty degeneration. Urate crystals were seen in the lumen of tubules and in intertubular areas (Fig. 5).

Electron microscopy of the pellet suspension obtained by ultracentrifugation (30,000 *g*) of the allanto-amnionic fluid of embryonated eggs inoculated with isolates B and SZ (Figs 6 and 7) revealed the presence of pleomorphic virions of coronavirus morphology, 95 to 120 nm in diameter and having 20 to 25 nm long surface projections.

Discussion

Virus isolation is of particular importance in IB, as demonstration of the causative virus is the most important evidence needed for diagnosing a disease showing itself in respiratory signs as IB. Isolation of IBV is known to be a rather difficult task. This is supported by the present study in which only 2 (B, SZ) out of 8 virus isolation attempts in 8 flocks succeeded.

The chances of virus isolation are the best at the initial, acute stage of IB. Both isolates B and SZ were obtained from such cases. Virus isolation attempts from birds with necropsy findings suggestive of chronic pathological alterations (blurred serous membranes, fibrin, adhesions) consistently failed. Rapid sample processing is especially important as IBV is sensitive to heat.

The test material must be treated with NDV antiserum which must not contain IBV antibodies. In one case even this treatment failed to eliminate NDV. For such cases Lomniczi and Stipkovits (1968) recommended to collect the allanto-amnionic fluid early, at PI hour 24, when the titre of NDV is still low and that of IBV has already come close to the peak.

Primarily the allanto-amnionic fluid collected from eggs showing embryo changes (even if collected on PI day 5–9) yielded virus on further passage.

Besides the morphological changes of the embryos, their gross pathological lesions may also be pathognostic. Swollen and urate-covered kidney are indicative of IB as evidenced by the presence of virions by electron microscopy (Fig. 4).

According to the cross-neutralization tests isolates B and SZ were neutralized in nearly identical dilutions by antiserum to strain M41 but not by antiserum to strain H52. Antisera to both isolates neutralized strains H52 in nearly identical dilutions. No close relationship was demonstrable between the two strains by virus neutralization test. A difference between the two isolates is that isolate B has higher antigenicity (its antiserum had a titre of 1 : 640 while that of isolate SZ a titre of $\geq 1 : 80$) and broader antigenic kinship (Table III).

These results indicate that the two isolates are IB viruses but differ from IBV reference strains M41 and H52. This was shown by the cross-neutralization tests in which even undiluted antisera to the reference strains failed to neutralize the two isolates, or neutralization took place only in low dilutions. Similar results were obtained when antisera produced against the isolates were tested against the reference strains.

The two isolates can be considered distinct IBV variants. This is the first report on the occurrence of such variants in Hungary. Further studies are needed to determine whether they should be reckoned with in IB control programmes.

In the physicochemical tests isolates B and SZ gave identical results with strain M41. Their physiochemical properties are the same as those reported for IBV by Lomniczi and Stipkovits (1968), Cowen et al. (1971) and Hofstad (1978). This is yet another evidence that the two agents are IB viruses.

Three passages of the two isolates and of the control strains M41 and H52 in chick embryo kidney cell cultures were needed for appearance of the typical CPE (rounded-off cells, formation of syncytia). Similar results were obtained by Churchill (1965), Otsuki et al. (1979) and Toro et al. (1987). The Beaudette

strain caused CPE already in the first passage. This was reported also by Churchill (1965). In cell cultures the isolates grew to a lower titre (by about 2 log) than in embryonated hen's eggs. The fact that serial passages were needed for adaptation of the isolates to cell cultures, together with the produced CPE, allows us to identify the two isolates as IBV.

The two agents isolated from 4- to 7-week-old broiler flocks experiencing respiratory signs were identified as IBV on the basis of morphological changes of the inoculated chicken embryos, the morphology of virions demonstrated in the kidney and in pellets concentrated by ultracentrifugation, the cross-neutralization and the physicochemical tests as well as the CPE produced in cell cultures.

Cross-neutralization tests with chicken sera showed that the isolates differ from the two reference strains (M41, H52) of IBV and can be considered distinct IBV variants. Their epizootiological role should be clarified by further studies.

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SANDWICH ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA) FOR MEASURING THE CONCENTRATION OF, AND DETECTION OF ANTIBODIES TO, AUJESZKY'S DISEASE VIRUS

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A double antibody sandwich enzyme-linked immunosorbent assay (ELISA) was developed for measuring Aujeszky's disease virus (ADV) antigen concentration and an inhibition technique based on the former was developed for detection of antibodies to ADV. The results were checked by determining the cytopathic and serum neutralization titres. The correlation was satisfactory in both cases, with correlation coefficients above 0.8. When measuring ADV antigen concentration, the lower limit of detection was 10^3 $TCID_{50}$/0.2 ml. The sensitivity of ELISA in detecting antibodies to ADV was found to be superior to that of the serum neutralization test and, thus, enabled the testing of rabbit and guinea-pig sera.

Keywords: Aujeszky's disease virus, detection, antigen concentration, antibody, sandwich ELISA, inhibition ELISA

Owing to the outstanding economic importance of Aujeszky's disease (AD) to the pig industry, its causative agent is often dealt with in the special literature. Numerous methods are known for the detection of Aujeszky's disease virus (ADV) and for measuring its antigen concentration: determination of the cytopathic titre in different cell culture systems (Peterson and Goyal, 1988), detection of ADV in infected organs and cell cultures by immunofluorescence (Narita et al., 1983; Mocsári and Süveges, 1985), plaque titration (McGregor et al., 1985), immunodiffusion and counterimmunoelectrophoresis (Williams et al., 1984), nucleic acid hybridization (Belák et al., 1987), etc. These techniques usually have variaties capable of detecting antibodies to ADV (Banks and Cartwright, 1983; Joo et al., 1984; Gutekunst et al., 1978; Papp-Vid and Dulac, 1979). Vaccine production, primarily the production of inactivated vaccines, requires an antigen concentration measuring method which is suitable for detecting both live and inactivated virus, is rapid, reproducible, and provides data that are in linear correlation with antigen concentration. The double antibody sandwich ELISA meets these requirements. No report on its use for detection of ADV can be found in the literature, though the technique is widely

used for the detection of other viral antigens (Edevag and Granström, 1986; Warford et al., 1986; Schorlemer et al., 1986).

Indirect ELISA and dot-enzyme immunoassay have long been used for quantitation of antibodies to ADV (Synder and Erikson, 1981; Erdei, 1985; Afshar et al., 1986). In spite of its simplicity and high sensitivity, these methods are not reliable enough. Nonspecific reactions due to different causes cannot be eliminated completely; they can at most be diminished by the use of highly purified virus and interpreted using a control antigen (Erdei et al., 1985). The so-called blocking ELISA (Sorensen and Lei, 1986) eliminates these problems when done with an immune serum of satisfactory quality; however, its sensitivity is poorer. Enzyme immunoassays using monoclonal antibodies have been also described (Van Oirschot et al., 1986); such antibodies, however, are not available in all laboratories. Development of the sandwich ELISA has permitted the use of an inhibition enzyme immunoassay which, though is more difficult to carry out than the indirect ELISA, gives much more reliable results. This is why such techniques have gained ground repidly in recent years (Torfason et al., 1988; Nielsen et al., 1987).

Materials and methods

Purification of virus for antiserum production

A 5-l suspension of ADV strain PHYLAXIA (strain collection of the National Institute of Hygiene, Budapest, Cat. No. 00236/82; infective titre: 10^6 $TCID_{50}$/0.2) grown on PK-15 cells (The American Type Culture Collection, Registry of Animal Cell Lines, 1972: CCL 33) was used as starting material. The suspension was frozen and thawed once, filtered through Whatman paper, then concentrated to 0.5 l in an Amicon ultrafiltration instrument. The virus was pelleted by ultracentrifugation in a Hitachi SCP 85H ultracentrifuge at $95{,}000 \times g$ for 2 h, then resuspended in 9.2 ml NTE buffer (1 mM EDTA, 50 mM TRIS, 0.15 M HCl, pH 7.4). From this material the virus was sedimented under a 30% (w/w) saccharose layer by ultracentrifugation as above, for 16 h. The pellet was resuspended in NTE buffer: 2.5 ml of virus suspension was obtained which contained 10.7 mg protein per ml as determined by Folin's reaction. The suspension was checked for purity by SDS-polyacrylamide gel electrophoresis (Laemmli, 1970). It was ascertained that the material worked well as indirect ELISA antigen. An uninoculated PK-15 cell suspension was frozen and thawed several times to destroy cell membranes and to release into the medium contaminating proteins of cellular origin. The material thus obtained was used for producing the control antigen in the same way as described for the test antigen.

Production of immune sera

The purified virus was diluted with PBS (0.14 M NaCl, 2.7 mM KCl, 6.4 mM Na_2HPO_4; pH 7.2) to a concentration of 100 μg/ml, inactivated with 0.2% ethylene imine at 37 °C for 24 h, and mixed to Freund's incomplete adjuvant at a ratio of 1 : 1. Five Californian White rabbits weighing 2.5 kg were inoculated intramuscularly with 1 ml, and 2 sheep intradermally, at 8 different injection sites, at each site with 0.1 ml of the inoculum. The animals were revaccinated four times at 2-week intervals. The virus-neutralizing titre of the obtained rabbit serum and sheep serum was 210 and 82, respectively.

Isolation of IgG fraction from the rabbit serum

This was done by the standard procedure (Harboe and Ingild, 1973), by ammonium sulphate precipitation and ion-exchange chromatography on DEAE-Sephadex A-50.

Purification of anti-ADV rabbit serum by affinity chromatography

The gel for affinity chromatography was prepared from 3 g of CNBr-activated Sepharose 4B and 200 mg of ADV-negative rabbit IgG as described previously (Kardi et al., 1988). Gel volume was 10 ml and bound protein concentration was 10.3 mg/ml. Five ml of sheep serum was applied on the column in one cycle and was washed down with 40 ml PBT (PBS supplemented with 0.05% Tween-20).

Indirect ELISA for checking the success of affinity chromatography

Anti-ADV rabbit IgG was diluted 1000-fold in carbonate buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, pH 9.6). One hundred μl volumes were measured into the wells of ELISA microtitre plates (Dynatech M 129A, Germany). The plates were incubated at 37 °C for 60 min, then washed with 200μl PBT per well for 3x3 min. From the purified and the untreated anti-ADV sheep serum dilution series from 10 to 80 were made in PBT-N containing 1% bovine serum albumin (BSA). The conditions of dosage, incubation and washing were as described for the first step. The anti-sheep peroxidase conjugate (Institut Pasteur, Paris) was diluted 8000-fold in PBT-N. One hundred μl volumes of this dilution were added to the wells, incubated for 30 min and washed as described above. Orthophenylene diamine (OPD) –H_2O_2 solution (34 mg OPD + 20 μl 33% H_2O_2 in 100 ml of buffer containing 50 mM Na_2HPO_4 and 25 mM citric acid, pH 5.4) was used as substrate. Two hundred μl volumes were measured into the wells and kept in dark room, at room temperature for 15 min. The reaction was stopped by adding 50 μl 2M sulphuric acid to each well. Extinctions were read in a Titertek Multiscan photometer at 492 nm.

Sandwich ELISA procedure

The conditions (dose of reagents, incubation, buffers, substrate) of the reaction were as described above. The optimal dilutions of reagents were determined by the usual checkerboard titration. Anti-ADV rabbit IgG used as catching antibody was applied in its 1 : 4000 dilution. The test samples were virus-containing cell culture supernatants taken during the process of vaccine production and its optimization procedures. From these samples a trebling dilution series consisting of 4 steps was made in PBT-N. The first step of the series was prepared by adding to 0.9 ml of the samples 0.1 ml buffer of tenfold NaCl and detergent concentration. The sheep serum purified by affinity chromatography and used as detecting antibody was used in a 2.5-fold dilution (i.e. 20-fold dilution of the starting material).

By interpolating the obtained extinctions we calculated that dilution of the virus-containing sample which gives an optical density (OD) value of 1. This was termed D_1.

Inhibition ELISA procedure

From the test sera 4-fold or 3-fold dilution series of 4 steps were prepared in PBT-N. The starting dilution was 1 : 5 for the rabbit and guinea-pig, 1 : 10 for the sheep and 1 : 20 for the pig serum. To these samples the 1 : 10 dilution of a non-purified suspension of ADV grown on PK-15 cells and concentrated to a D_1 value of 45 was added at a ratio (v/v) of 1 : 1. In the case of pig sera antigen-free, so-called "blind" dilutions were also made. The mixtures were incubated at 37 °C for 3 h, then at 4 °C overnight. The following day the concentration of nonreacted antigen was measured without dilution by sandwich ELISA.

By interpolating the dilution–extinction correlations found for the test sera, we determined the dilution ($D_{1/2}$) which gave an optical density corresponding to 50% of that obtained for the sample containing the negative serum.

Determination of the cytopathic and virus-neutralizing titres

The $TCID_{50}$ was determined in primary calf testicle cell cultures in the usual way. For antibody level determinations Vero cells (ATCC, CCL 81) were used. Evaluation was done by Karber's method (Dömök and Ruzicska, 1865; Kucsera, 1966).

Results

Sheep anti-ADV immune serum used in the sandwich ELISA as detecting antibody gave a disturbing background reaction with anti-ADV rabbit IgG used as "catching" antibody. This background reaction was eliminated through

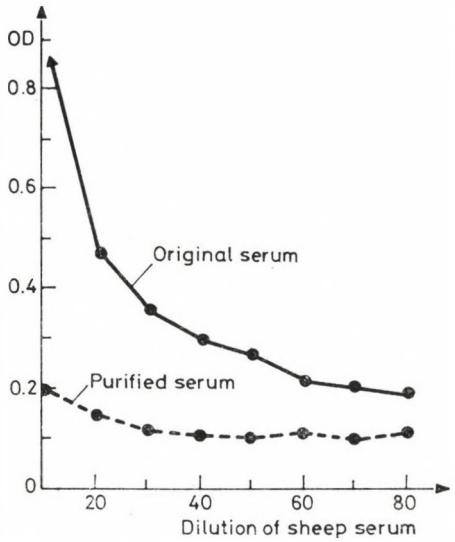

Fig. 1. Indirect ELISA for checking the success of affinity-chromatographic purification of anti-ADV sheep serum

purification by affinity chromatography. The success of the purification process was checked by indirect ELISA which was interpreted as shown in Fig. 1. It can be seen that in the 20-fold dilution used by us the extinction indicating non-specific reaction decreased from 0.45 to 0.15. All media (cell culture supernatants of different origin and history) used for determining ADV concentration were checked for any possible positive reactions of their components in our system ($D_1 > 0$). No such positive reactions were found.

The measured values, expressed in D_1, were compared with the infective virus titres of a total of 170 virus suspensions. The samples were grouped by $TCID_{50}$ values and from the D_1 values means $\pm$ standard deviations were calculated. The obtained correlations are shown in Fig. 2.

The same data were grouped by cell culture in which the test virus suspensions was grown (PK-15, BHK-21 (ATCC, CCL 10) or Vero). From infective virus titres and ELISA results obtained for the 3 groups, data of the regression line were determined (Fig. 3).

Figure 4 shows the typical time-course of virus growth on PK-15 cells (microcarrier). By ELISA peak antigen concentration occurred 20 h later than by infective titre determination.

Antibody levels to ADV were measured first of all in pig sera, but rabbit, guinea-pig and sheep sera were also used. The $\log_4$ dilution-extinction correlation obtained is presented in Fig. 5. The anomalous course obtained for some pig sera was due to the fact in the the sandwich ELISA these samples give positive reaction even without the addition of antigen. The resulting extinction

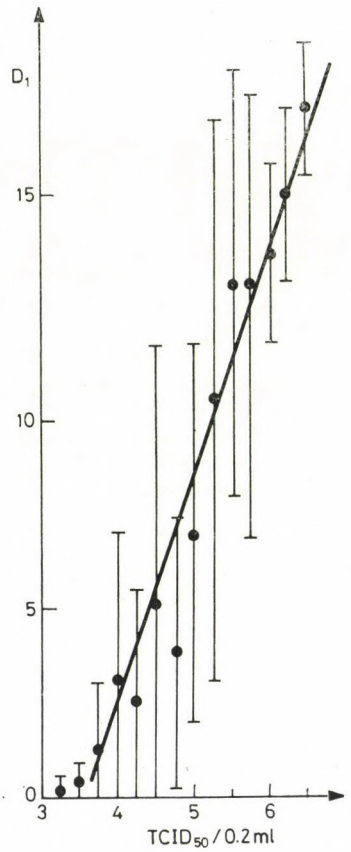

Fig. 2. Determination of the concentration of Aujeszky's disease virus: comparison of infective titre determination and ELISA

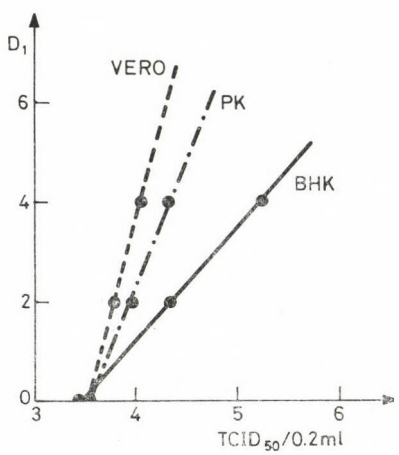

Fig. 3. Correlation of the infective titre and the ELISA result of Aujeszky's disease virus suspensions grown on different cells. BHK-21: m =2.21; r = 0.96; PK-15: m = 6.71; r = 0.97; Vero: m = 8.55; r = 0.93

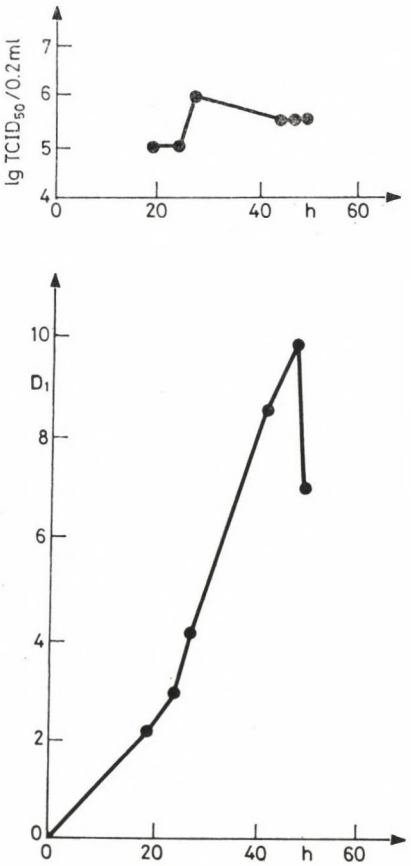

Fig. 4. Growth of Aujeszky's disease virus in microcarrier cultures of PK-15 cells

increment declines with the dilution of serum, while the concentration of non-reacted antigen remaining after the inhibition increases. This is how the minimum curve shown in the figure emerges. Such a phenomenon was not observed for sera from other species of animals and it occurred in only a few pig sera, usually in those containing the highest antibody levels. The problem was eliminated by the parallel use of antigen-free, so-called "blind" dilutions.

The virus-neuralization and ELISA results are compared in Fig. 6. Forty-five pig, 10 sheep, 10 rabbit and 5 guinea-pig sera were tested (total: 70 serum samples). Pig and sheep sera had come from naturally infected and vaccinated herds of flocks of different farms. Rabbit and guinea-pig sera were obtained during the testing of experimental vaccines.

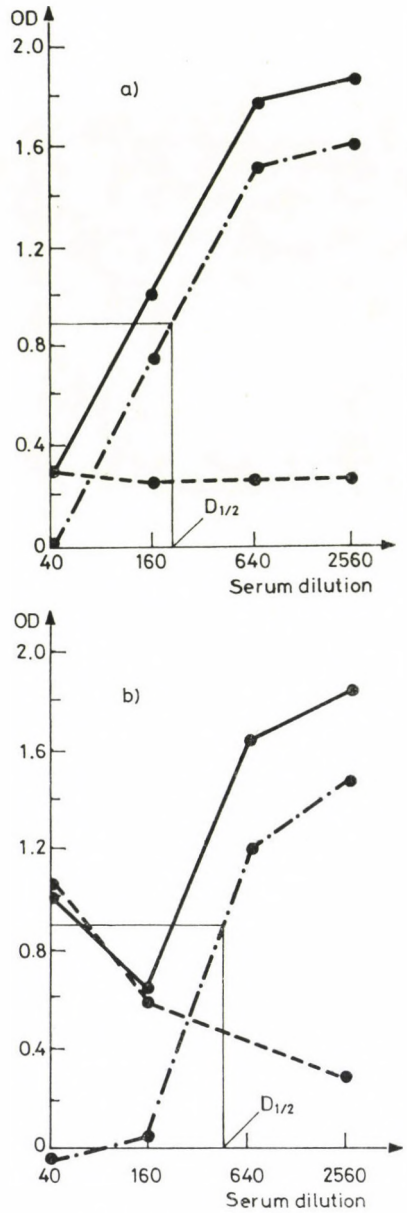

Fig. 5. Testing of pig sera by inhibition ELISA. (a) Normal course; (b) Abnormal (anomalous) course. Solid line: dilution series with antigen added; broken line: dilution series without antigen; dotted line: difference of the two. Negative value used for calculating $D_{1,2}$: OD = 1.8

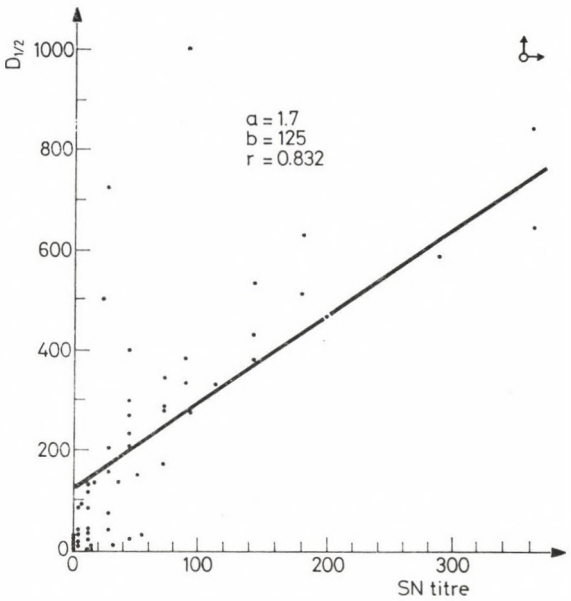

Fig. 6. Comparison of inhibition ELISA results and virus-neutralizing titres in detection of antibodies to Aujeszky's disease virus

Discussion

The results of the sandwich ELISA and infective titre determinations are compared in Fig. 2. The correlation can be well approximated with a line: up to 10^5 $TCID_{50}$/0.2 ml, however, the exponential character is evident. This is due to the fact that the sandwich ELISA shows a linear whereas the cytopathic titre shows a logarithmic correlation with the antigen concentration. The ELISA is less sensitive: its lower limit of detection is around 10^3 $TCID_{50}$/0.2 ml. Highly reproducible results were obtained above $10^{3.5}$ $TCID_{50}$/0.2 ml. This does not pose a problem in the practice, as in vaccine production always higher virus activities are used. At the same time, the limitations of comparing the two methods are evident, too. Standard deviation is rather high, though the ELISA results show fairly good reproducibility. Testing the same sample several times, $> 20\%$ differences between the D_1 data were rare.

The differences found between the ELISA results and the cytopathic titres primarily arise from the dissimilar principle of the two methods, i.e. from the fact that the cytopathic titre only includes live virus concentrations while the ELISA result refers to total antigen content. The higher sensitivity of infective titre determination, a finding which is in virtual contradiction to the above statement, may be explained as follows: while in a suitable cell culture system even a single viable viral particle can grow to high titre and cause

visible changes, for the colour reaction detectable by ELISA a higher virus antigen concentration is needed.

A more detailed analysis of the above data is shown in Fig. 3. The test samples were virus suspensions grown in 3 different cell cultures. Titration was consistently done on primary calf testicle cells. The cytopathic titre and total antigen concentration measured by ELISA were in a fairly high linear correlation for all three cell cultures. The lines intersect the $TCID_{50}$ axis around 3.5; however, they markedly differ in steepness. To a given infective titre rather dissimilar D_1 values belong on the different cell types, as the proportion of viable virions within total virus concentration is different. Figure 4 can be interpreted in the same way: within a growth cycle the antigen concentration still rises while changes of the medium already cause a reduction in live virus titre. As the immunogenicity of an inactivated vaccine depends on the concentration of virus antigen contained in it rather than on viability of the virus, the conditions of vaccine production should be chosen on the basis of the ELISA results rather than the infective virus titres. The inhibition ELISA is by all means more suitable for specific detection of antibodies to ADV than the generally accepted indirect ELISA. As our sandwich ELISA system does not detect contamination present in the virus suspension, the measured reduction in extinction is exclusively due to the reaction between virus and antiviral antibody. Consequently, purified antigen is not needed for this test. A further advantage of the inhibition method is that sera from different species of animals can be tested under identical conditions, in the same system: this is especially important in the evaluation of experimental vaccines. Such optimization of an indirect ELISA would be difficult to carry out because of the widely varying antibody levels and, first of all, the very dissimilar nonspecific reactions.

A comparison of our results with the virus-neutralizing titres is presented in Fig. 6. The coefficient of correlation is 0.83 which meets the requirements of linearity. The ELISA is more sensitive. The axis intercept is 125, indicating that neutralization gives negative results up to $D_{1/2} = 125$. The figure shows that the difference in sensitivity between the two methods is not in the least so large; however, in the low antibody level range the correlation strongly deviates from linear. At $D_{1/2} < 15$ virus neutralization does not work. Although that range has low significance when testing pig sera, it is important in vaccination trials using rabbits and guinea-pigs.

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