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INFLUENCE OF OXYTOCIN AND PROSTAGLANDIN ON SEMEN CHARACTERISTICS AND PROCESS OF EJACULATION IN BUFFALO BULLS

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(Received September 1, 1987)

The effect of oxytocin and prostaglandin on ejaculate characteristics of bulls was studied. One ejaculate each was collected from four bulls after injecting them intramuscularly with saline, and used as control. Different doses of oxytocin (G. Richter Pharmaceutical Works, Budapest; 0, 5, 10 and 20 IU; Experiment 1) and prostaglandin $F_{2\alpha}$ (Enzaprost, Chinoin, Budapest; 0, 0.5, 1 and 2 mg; Experiment 2) were injected intramuscularly in a series of 4×4 Latin squares in both treatments, separately, that is 36 samples altogether. One ejaculate was collected and evaluated after each injection. Twelve semen samples from bulls treated with 20 IU oxytocin, 2 mg prostaglandin and non-treated controls were extended with freshly prepared yolk citrate buffer and cooled at 5 °C for four days (Experiment 3). Subsequently, the survival rate was examined daily. A comparative study was also performed to examine the *in vitro* supplementation of semen samples with oxytocin and prostaglandin ($n = 3$). The samples were extended as mentioned above (Experiment 4). In bulls treated with prostaglandin and oxytocin no significant difference was found in the number of spermatozoa per ml, but ejaculate volume and the number of motile sperm/ejaculate were significantly ($P < 0.05$) increased. The concentration of spermatozoa was significantly increased after prostaglandin treatment alone. No significant increase in sperm output was obtained after injection of oxytocin. Spermatozoa were extended at 5 °C for four days. The ejaculates collected from bulls pretreated with both oxytocin and prostaglandin showed no significant difference from the control. Semen samples supplemented *in vitro* with both hormones (20 IU oxytocin and 2 mg prostaglandin/ml of pooled semen) showed a significant difference ($P < 0.05$) from the control. The results are discussed as a possible means of improving the bulls' semen output and sperm survival rate.

Keywords: Oxytocin, prostaglandin, semen characteristics, ejaculate, motile, sperm, semen, output, buffalo bull.

The evidence for oxytocin release at coitus in the female suggested a possible parallelism in the male (Bell et al., 1975). In the blood plasma of male animals a 3- or 4-fold increase in oxytocin activity was found after genital stimulation immediately before and shortly after service (Fitzpatrick, 1966). Ewy et al. (1972) and Barowiez et al. (1972) found that during semen collection oxytocin activity was increased in the plasma of boars. The highest level was measured during the final phase of ejaculation.

In their experiments on rabbits Fjellstrom et al. (1968) obtained indirect evidence that oxytocin stimulated the accessory sex glands. Bereznev (1963) reported that oxytocin stimulated contractions of the epididymis and deferent

duct in bulls. Also, norepinephrine, acetylcholine, prostaglandin and oxytocin were found to produce an increase in the contractility of the tunica albuginea, consisting mainly of an increase of tonicity. Ellis et al. (1980) and Mohy El-Deen (1984) found that oxytocin stimulated contractility of the seminiferous tubules and testicular capsule of the rat and mink. Contractility of the seminiferous tubules facilitates sperm release and flow as well as sperm transport through the testis into the epididymis.

El-Azab et al. (1974), Knight (1974), Sharma and Hays (1976), Klenner and Boryczko (1980) and Alkass and Ibrahim (1984) found that injection of oxytocin immediately before ejaculation caused an increase in the number of spermatozoa per ejaculate and also in semen volume in the bull, rabbit and ram. Several attempts have been made to improve sperm transport and fertility by injecting animals with hormones. The significance of prostaglandin in sperm transport has been demonstrated in rabbits and bulls (Baader and El-Keraby, 1982).

On large animals, especially buffaloes, the influence of such hormones on sperm behaviour has been little studied.

The objective of the present work was to study the influence of treatment with oxytocin and prostaglandin (together or separately) on sperm output, motility and survival at 5 °C.

Materials and methods

Animals

Four buffalo bulls, provided by the Faculty of Agriculture, Kafr El Sheikh Experimental Station, were used. The average body mass of the bulls was 600 kg and their age was three years on the average. The animals were reared, fed and treated according to the routine management system used at the station. They were free of diseases, had a healthy appearance and normal semen characters. All the animals were sexually mature.

Semen evaluation

A single ejaculate was collected from each bull in both experiments (experiments 1 and 2). All ejaculates were examined for ejaculate volume, percentage of sperm showing progressive motility, and sperm concentration ($\times 10^9$), total number of motile sperm/ejaculate ($\times 10^9$) (parameters studied in experiments 1 and 2).

Experiment 1

Different doses of oxytocin (0, 5, 10 and 20 IU) were administered intramuscularly. The interval between oxytocin injection and ejaculate collection

was at most 10 min. All the animals were stimulated sexually, using three mounts, before hormonal treatment and semen collection. The number of samples was 16.

Experiment 2

The same design was used as in experiment 1, with the exception that different doses of prostaglandin (0, 0.5, 1 and 2 mg) were injected intramuscularly.

Experiment 3

Semen samples collected from each bull in experiments 1 (n = 16) and 2 (n = 16) were diluted and extended with freshly prepared yolk citrate buffer. Freshly collected normal semen samples (n = 4) were extended with the same yolk citrate buffer without any supplement and used as control. The extender contained 2.9% sodium citrate (dehydrated), 20% egg yolk, 500 mcg streptomycin and 500 IU penicillin per ml. The average dilution rate was 1 : 10 for all the treatments.

The samples were refrigerated at 5 °C for four days. Duplicate subsamples were taken daily and examined for sperm motility at a magnification of ×300.

Experiment 4

Pooled semen samples were obtained from four buffalo bulls and distributed into three plastic vials (n = 3). To the first vial 20 IU oxytocin/ml semen, while to the second 2 mg prostaglandin $F_{2\alpha}$/ml semen was added. The third vial served as control. The same extender was used as in experiment 3, and the average dilution rate was 1 : 10 for all the three samples. The samples were refrigerated at 5 °C and were examined daily for sperm motility at a magnification of ×300, as in experiment 3.

A comparative study was performed between experiment 3 and experiment 4 as regards the survival of sperm motility during preservation.

Statistical analysis was carried out as described by Snedecor and Cochran (1971).

Results

Semen characteristics obtained for buffalo bulls injected with different doses of oxytocin or prostaglandin are shown in Table I. The applied doses exerted a significant effect on some of the traits studied. Injection of 5, 10 or

Table I

Effect of different doses of oxytocin and prostaglandin on semen quality and sperm output in buffalo bulls

Semen characteristics	Oxytocin, IU/animal			
	0	5	10	20
Semen volume, ml	2.41 ± 0.25^a	2.80 ± 0.28^b	3.40 ± 0.38^c	3.80 ± 0.12^d
Sperm concentration, $\times 10^9$/ml	1.13 ± 0.11^a	1.18 ± 0.32^a	1.15 ± 0.22^a	1.12 ± 0.20^a
Sperm concentration, $\times 10^9$ per ejaculate	2.69 ± 0.76^a	3.31 ± 0.87^a	3.93 ± 0.11^a	4.29 ± 0.88^a
Sperm motility, %	86.30 ± 4.78^a	82.50 ± 6.45^a	86.25 ± 4.78^a	87.50 ± 5.00^a
No. of motile sperm per ejaculate, $\times 10^9$	2.31 ± 0.12^a	2.81 ± 0.87^b	3.43 ± 1.11^c	3.78 ± 0.93^d
% increase of sperm output/ejaculate	—	23	46	57
% increase of ejaculate volume	—	16	41	58
% increase of motile sperm/ejaculate	—	21	48	64

Semen characteristics	Prostaglandin, mg/animal			
	0	0.5	1	2
Semen volume, ml	2.50 ± 0.37^a	3.00 ± 0.14^b	3.50 ± 0.29^c	3.90 ± 0.37^d
Sperm concentration, $\times 10^9$/ml	1.15 ± 0.25^a	1.11 ± 0.19^a	1.19 ± 0.26^a	1.18 ± 0.22^a
Sperm concentration, $\times 10^9$ per ejaculate	2.92 ± 0.99^a	3.31 ± 0.53^b	4.41 ± 1.03^c	4.61 ± 1.10^d
Sperm motility, %	85.00 ± 7.07^a	83.75 ± 4.80^a	87.50 ± 2.88^a	87.50 ± 0.50^a
No. of motile sperm per ejaculate, $\times 10^9$	2.46 ± 0.86^a	2.79 ± 0.40^b	3.85 ± 0.87^c	4.00 ± 0.75^d
% increase of sperm output/ejaculate	—	14	51	58
% increase of ejaculate volume	—	20	40	56
% increase of motile sperm/ejaculate	—	13	56	63

$^{a, b, c, d}$ Mean values with different superscripts are significantly different ($P < 0.05$). Each value represents the mean of four buffalo bulls

20 IU oxytocin and 0.5, 1 or 2 mg prostaglandin significantly increased the semen volume ($P < 0.05$). The mean values of percentile increase due to treatment were 16.0, 41.0 and 58.0% and 20.0, 40.0 and 56.0% for the different doses of oxytocin and prostaglandin, respectively.

No significant difference was found in the number of spermatozoa per ml and sperm motility after injection of oxytocin or prostaglandin, as compared to the control samples. The total number of spermatozoa per ejaculate (sperm output) was in a positive correlation with the doses injected in both treatments. However, significant ($P < 0.05$) correlations were obtained only for the prostaglandin treatment (14, 51 and 58% increase after the injection of

0.5, 1 and 2 mg prostaglandin, respectively). The increase in sperm output was large but not statistically significant in the case of oxytocin treatment (23.0, 46.0 and 57.0% increase in sperm output after the injection of 5, 10 and 20 IU oxytocin, respectively).

The overall means of increase in motile sperm per ejaculate were 21.0, 48.0 and 64.0% and 13.0, 56.0 and 63.0% for oxytocin- and prostaglandin-treated animals, respectively. The differences were in a positive correlation

Table II

Sperm storability after injection of buffalo bulls with prostaglandin and oxytocin before semen collection

Duration of storage at 5 °C (h)	Control	Prostaglandin $F_{2\alpha}$	Oxytocin
	Survival rate, %		
1	67.5 ± 5.9	68.5 ± 4.1	67.0 ± 4.2
24	45.5 ± 5.5	45.0 ± 5.3	44.5 ± 5.5
48	33.5 ± 4.7	34.5 ± 4.4	34.0 ± 3.9
72	26.5 ± 2.4	26.5 ± 3.4	27.0 ± 2.6
96	18.5 ± 4.7	18.5 ± 5.8	18.0 ± 4.8
Mean, $\bar{x}$	38.3 ± 19.0^a	38.6 ± 19.3^a	38.1 ± 18.8^a
Σx	191.5	193.0	190.5
Σx^2	8793.25	8952.00	8678.25

$^{a, b, c}$ Mean values with different superscripts are significantly different ($P < 0.05$). Each value represents the mean of four buffalo bulls

Table III

Effect of adding prostaglandin or oxytocin to the semen extender on the storability of buffalo bull spermatozoa

Duration of storage at 5 °C (h)	Control	2 mg prostaglandin/10 ml diluted semen	20 IU oxytocin/10 ml diluted semen
	Survival rate, %		
1	64.5 ± 7.9	72.0 ± 7.5	67.0 ± 4.8
24	47.0 ± 6.7	55.5 ± 2.9	49.5 ± 6.0
48	37.0 ± 4.8	44.5 ± 6.4	40.5 ± 6.4
72	29.0 ± 5.6	39.0 ± 6.2	33.5 ± 7.1
96	20.0 ± 7.8	30.5 ± 5.5	23.5 ± 7.8
Mean, $\bar{x}$	39.5 ± 17.0^a	48.1 ± 16.0^b	42.7 ± 16.0^c
Σx	197.5	241.5	213.5
Σx^2	8979.25	12,695.75	10,204.75

$^{a, b, c}$ Mean values with different superscripts are significantly different ($P < 0.05$). Each value represents the mean of four buffalo bulls

with the doses applied and were significant ($P<0.05$). The survival rate and storability of buffalo bull spermatozoa collected after prostaglandin or oxytocin injection are given in Table II. The results show that injection of prostaglandin and oxytocin did not cause significant differences in the survival rate and storability of sperm, as compared to the control samples. The motility of spermatozoa was similar to that found for the control samples. The survival rate of semen samples supplemented *in vitro* with 20 IU oxytocin or 2 mg prostaglandin is summarized in Table III. Mean sperm motility was 39.5, 48.1 and 42.7% for control samples, prostaglandin and oxytocin treatments, respectively. The differences are significant ($P < 0.05$). The addition of prostaglandin or oxytocin to semen extenders significantly improved sperm motility. The mean sperm motility obtained for prostaglandin supplementation was always superior and improved the survival of spermatozoa during semen storage at 5 °C throughout the four days of storage more than did oxytocin treatment. Analysis of variance showed that the mean motility percentage of spermatozoa diluted with an extender containing 2 mg prostaglandin or 20 IU oxytocin had a significant improving effect on the survival rate of buffalo bull spermatozoa.

Discussion

The results of experiments 1 and 2 clearly demonstrate the effect of prostaglandin or oxytocin injection on semen volume. Combined data from these two experiments show a 29.0 and 38.6% increase in ejaculate volume, a 42 and 41% increase in the total number of sperm per ejaculate, and a 44.3 and 44.0% increase in motile sperm, as compared to the control. After injecting the bulls with prostaglandin or oxytocin, the rise in the number of spermatozoa per ejaculate was attributable to an increase in both semen volume and number of sperm per ml (sperm concentration). Copulation raises the oxytocin content of the blood, and the male ducts contain contractile tissues sensitive to hormone treatment. The results of the present work are consistent with data reported by Knight (1974), El-Azab et al. (1974), Sharma and Hays (1976) and Alkass and Ibrahim (1984) who found that oxytocin might influence the secretion rate of the male accessory sex glands. This was reflected in the increase of ejaculate volume. It is logical to suggest that oxytocin or prostaglandin may play an important role in ejaculation, a process probably depending on reflexes arising during coitus. Data obtained so far indicate that under certain conditions oxytocin may affect the male duct system, resulting in increased sperm passage rate. Evidence obtained during the present study suggests that prostaglandin injection has a positive effect on the rate of sperm passage and on sperm output. Previous results indicated that, when

administered immediately before semen collection, oxytocin increased sperm concentration and ejaculate volume in a similar degree as the injection of prostaglandin $F_{2\alpha}$, while prostaglandin was more potent in increasing the rate of sperm passage in the male ducts and had a stronger effect on the secretion of the accessory sex glands. Smooth muscle is known to be present in the limiting membrane of the seminiferous tubules and can be activated *in vitro* by oxytocin or prostaglandin (Sharma and Hays, 1976). On the other hand, several investigators reported results contradicting the above findings. Biellanski and Tischner (1968) reported that the total daily output of spermatozoa was not affected significantly by oxytocin injection. This is in full agreement with our findings obtained for oxytocin treatment in the present study.

El-Azab et al. (1974), Beneit et al. (1980) and Botting (1981) found no evidence that oxytocin stimulated contraction or secretion in the accessory sex glands in male goat, bull and ram. The results obtained in this study show that the male reproductive system of buffalo bulls responds to the administration of oxytocin or prostaglandin. This is very important in order to collect a good ejaculate volume from buffalo bulls. Results of the present study, obtained on the effect of hormone supplementation of semen extenders, are in full agreement with those of Chang et al. (1973), Marley et al. (1976), Gwllory et al. (1979) and Baader and El-Keraby (1982) who found that sperm transport and fertility could be improved by administering hormones to animals, or by adding them to the extenders. On the other hand, we found that animals injected with hormones showed no improvement in sperm survival rate and storability at 5 °C. Only a few studies have been done on the influence of such hormones on bull spermatozoa *in vitro*, and research on this subject has been completely lacking for the sperm of buffalo bulls. The present findings show that the sperm of buffalo bulls responds to such hormone supplementation of the extenders. We can conclude that buffalo sperm can be improved and sperm output can be increased under practical conditions by the treatments administered, a fact essential for artificial insemination in buffaloes.

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SOME IMMUNE REACTIONS INDUCED IN PIGS BY AN ATTENUATED AUJESZKY'S DISEASE VACCINE

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Ten pigs aged 3 months were immunized once with a live vaccine containing attenuated Aujeszky's disease virus (AyV). Two pigs each were killed by bleeding on days 4, 7, 14, 21 and 28 after vaccination, and samples were taken from their blood, tissues and organs.

By immunofluorescence (IF) viral antigen was demonstrable at the site of injection up to day 7. In the lymph node and spleen samples the antigen persisted up to day 21. In the tonsils it was demonstrable only on day 7, while all the spinal cord, brain, and intervertebral ganglion samples were negative.

Circulating immune complexes (IC) were demonstrable in the peripheral blood already on day 4, before the virus-neutralizing (VN) antibodies appeared. The IC persisted up to day 14. The tissue samples did not contain deposited IC.

Up to postvaccination day 4, immunomorphological changes characteristic of the inductive stage, accompanied by enhanced phagocytic activity and appearance of secondary follicles, developed in the lymphoid organs.

Sensitization of the organism was indicated by the lymphocyte-stimulation test (LST) and the immunorosette formation test from the 7th and 4th day, respectively. Antibody-dependent cytotoxic reactions measured on xenogenous target cells were detectable from days 4–7 onward. The results furnish evidence that, in addition to the VN antibodies, the cell-mediated immune response plays an important role in the development of immunity following vaccination.

Keywords: Aujeszky's disease, pig, humoral immune response, cell-mediated immune response, immunomorphological changes, immune complexes.

In recent years it has been proved that, besides the humoral immune response, cell-mediated immune reactions have a decisive role in the development of immunity against herpesviruses, including Aujeszky's disease virus (AyV; Davies and Carmichael, 1973; Rouse and Babrick, 1974; Russel, 1974; Rosenberg and Notkins, 1974; Smith and Mengeling, 1977; Rottinghaus et al., 1978; Gutekunst, 1979; Ashworth et al., 1979; Wittmann et al., 1985*a*). Little is known about the cell-mediated immune reactions induced by vaccines used in the immunoprophylaxis of Aujeszky's disease (Bearan et al., 1980; Oirschot and Gielkens, 1984; Wittmann et al., 1985*b*), and the factors having a role in their regulation have also remained unclear.

In the present experiments we studied, in addition to the persistence of the attenuated AyV vaccine virus antigen, the immunomorphological changes

occurring in the different organs and tissues, the kinetics of formation of circulating immune complexes (IC), and the development of humoral and cell-mediated immune responses.

Materials and methods

Experimental animals. Twelve Large White pigs, aged 3 months and obtained from an Aujeszky's disease free herd, were used. Ten of the pigs received a single 1-ml dose of an attenuated AyV vaccine containing the Bartha strain (Bartha, 1961), intramuscularly. Two pigs served as control.

Two pigs each were killed by bleeding on days 4, 7, 14, 21 and 28 after vaccination. Blood samples were taken from the experimental animals in heparinized tubes and in those without anticoagulant. Samples were taken from the site of inoculation, from the regional lymph nodes, spleen, tonsils, lumbar segment of the spinal cord, and brain stem, for histological and electron-microscopic examination. Cryostat sections were prepared from the tissue samples for immunofluorescence (IF). The reactions of leucocytes separated from the spleen, lymph node and blood samples were examined by *in vitro* cellular tests. The two control pigs were killed by bleeding on day 4 and 14, respectively, and sampled as above.

Aujeszky's disease virus antigen. Virus suspension of 10^8 $TCID_{50}$/0.1 ml concentration, obtained from pig kidney cell monolayers, was purified mechanically and, subsequently, treated with a detergent mixture containing sodium deoxycholate, TRITON-X and beta-mercaptoethanol (Medveczky and Tuboly, 1981).

Virus neutralization (VN) test. The VN test was performed as described in manuals.

Lymphocyte separation. Leucocytes were separated from heparinized blood samples with Ficoll Paque. Cell density was adjusted to 10^6/ml in Hanks solution containing 10% fetal calf serum. The spleen and lymph node samples were suspended in PBS in a glass homogenizer. From the cell suspensions the leucocytes were separated similarly as from the blood samples, and resuspended to give a density of 5×10^6 cells/ml. 1.5-ml volumes of the cell suspensions were measured into Leighton tubes, and antigen solutions were added as required in the different tests.

Lymphocyte stimulation test (LST). To each cell suspension 0.1 ml AyV antigen solution was added, and the rate of blastogenesis was determined by autoradiography, on the basis of ^{3}H-TdR incorporation, as described earlier (Kiss and Tuboly, 1981).

Immunorosette formation (RCF). After pretreatment with tannic acid, a 1% sheep red blood cell (SRBC) suspension was treated with an equal

volume of antigen solution, then 0.1-ml volumes were added to the lymphocyte suspensions in the Leighton tubes. The proportion of rosette-forming cells was read microscopically (Friemel, 1980).

Cytotoxic (CT) reaction. SRBCs that had bound AyV antigen served as target cells. The reaction was carried out as described previously (Kiss and Tuboly, 1981), with the modification that each sample was supplemented with 50 μl of tenfold diluted anti-AyV serum of 1 : 8 titre.

Detection of circulating immune complexes (IC). To detect IC in the blood serum, a modification (Mayr et al., 1982) of the precipitation test performed with polyethylene glycol (PEG) 6000 (Haskova et al., 1978) was applied. Spectrophotometry was performed at 450 nm; a 10% aggregated sheep IgG solution treated in a waterbath of 63 °C for 30 min was used as standard. The presence of viral antigen or IgG in the precipitate was assessed by ELISA (Nel and Stevens, 1980) as follows: the precipitate was first washed in 3.75% PEG-6000 solution, then dissolved in PBS (pH 7.2). Anti-AyV serum and anti-pig peroxidase conjugate (HUMAN, Budapest) were used as immune reagents.

Histological and electron-microscopic examinations. The tissue samples were embedded in paraffin (Tuboly et al., 1984). For electron microscopy, pieces of appropriate size from the spleen and upper cervical lymph node of two pigs each, killed by bleeding on day 4 and 14, were fixed in 2.5% glutaraldehyde buffered with sodium cacodylate, then in 1% osmium tetroxide. Sections from Durcupan-embedded samples were cut with an OMU-3 "Reichert" ultramicrotome, counterstained with uranyl acetate and lead citrate, and examined in a Philips 201-CS electron microscope.

Immunofluorescence (IF) tests. A direct IF method was used to detect virus and viral antigens. Cryostat sections of the tissue and organ samples were incubated for 60 min with anti-AyV conjugate (Bioveta, Czechoslovakia), washed in PBS for 3×10 min, covered with a 1 : 3 mixture of glycerol and PBS, and a coverslip was placed onto them. The test was read with an NU-2 Zeiss microscope.

The same tissue and organ samples were tested for deposited IC as follows: the samples were covered with anti-pig IgG FITC conjugate (Miles, Wien), then treated and evaluated as described above.

Results

The detectability of AyV antigen in the tissue and organ samples by IF is shown in Table I.

Due to viral antigen accumulated on the surface of macrophages, the whole, or part of the, cell surface exhibited granular fluorescence. In the

Table I

AyV antigen demonstrable by the IF test at the site of injection and in the different organs

Sites and organs	Postvaccination days				
	4	7	14	21	28
Site of injection	+++	++	—	—	—
Lymph node	+++	++	++	+	—
Spleen	++	++	++	+	—
Tonsils	—	+	—	—	—
Spinal cord	—	—	—	—	—
Brain stem	—	—	—	—	—

Table II

VN antibodies and IC in the blood serum

	Postvaccination days				
	4	7	14	21	28
VN antibodies	—	—	1 : 2	1 : 4	1 : 4
IC,	+	+	+	—	—
mg/ml	0.070	0.100	0.020	0.00	0.00

IC = circulating immune complex (equivalent to 10% aggregated porcine IgG)

regional lymph node the "positive" macrophages occurred in groups in the paracortical zone (Fig. 1) or, occasionally, dispersed in the cortex. In the spleen the viral antigen bound to macrophages was seen in groups around the follicular centres or dispersed in the parenchyma; in the tonsils it showed a similar distribution.

Data on the appearance and persistence of circulating IC, along with the VN titres, are given in Table II. No deposited IC were demonstrable by IF in cryostat sections of the tissue and organ samples tested.

The results of the LST and RCF test are shown in Text-figs 1 and 2, respectively.

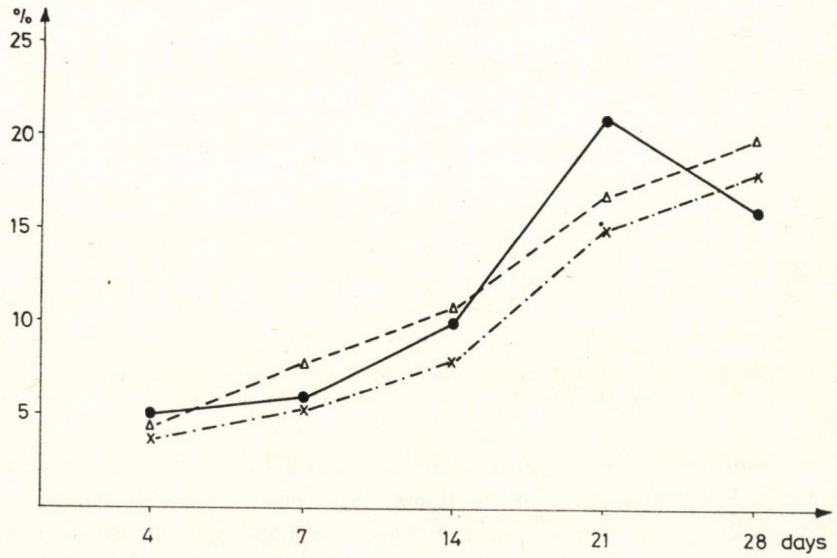
A comparison of the LST results has revealed that the stimulation index of blood lymphocytes reached the maximum on day 21, and decreased subsequently. On the other hand, the blastogenic transformation of spleen and lymph-node lymphocytes increased up to day 28.

The results of the RCF test are about the same either blood or spleen lymphocytes are examined. However, the lymphocytes of lymph node origin bound about 50% less antigen-sensitized red blood cells on their surface on days 21 and 28.

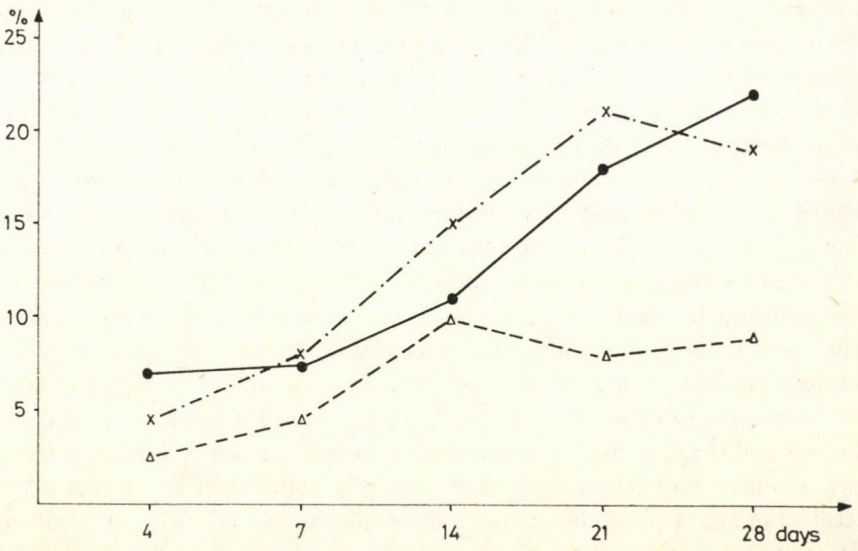
The spectrophotometric data of CT reactions of blood, spleen and lymph-node lymphocytes are given in Text-fig. 3. On day 4, blood lymphocytes had

detectable cytotoxicity in a xenogenous system: by day 7 this decreased to 0 and increased again up to day 28. Spleen and lymph-node cells lysed the target cells only after day 7. The control samples were negative.

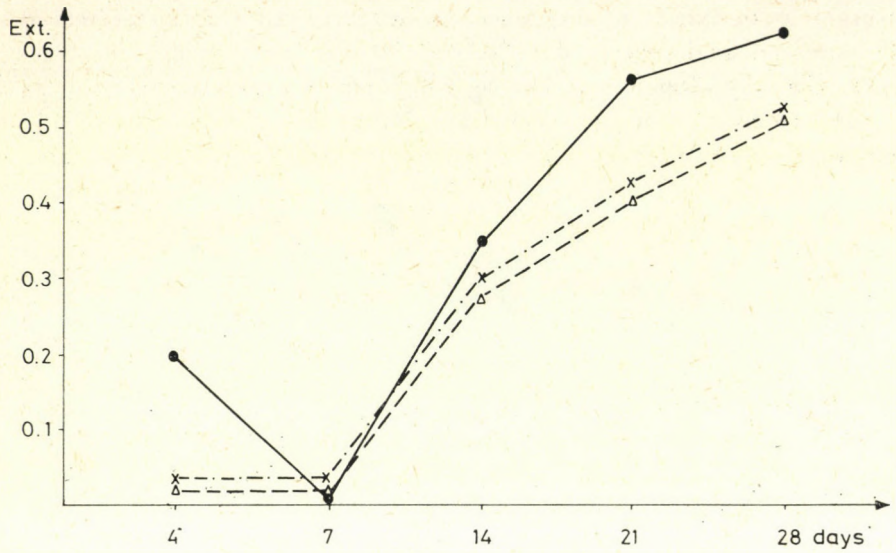
Histologically, on postvaccination days 4 and 7 at the site of injection there was a serous-haemorrhagic infiltration extending to the corium, sub-



Text-fig. 1. Results of the lymphocyte-stimulation test (solid line: blood; broken line: lymph node; dotted line: spleen)



Text-fig. 2. Results of the RCF test (solid line: blood; broken line: lymph node; dotted line: spleen)



Text-fig. 3. Results of the antibody-dependent cytotoxic (CT) reactions (solid line: blood; broken line: lymph node; dotted line: spleen)

cutaneous connective tissue, and muscles. The infiltration consisted of mononuclear cells, polymorphonuclear inflammatory cells, and an increasing proportion of fibroblasts. The perimysium of the surrounding muscle tissue contained proliferative infiltration, focal or diffuse, mainly consisting of lymphocytes, lymphoblasts and histiocytes (Fig. 2). Part of the histiocytes had swollen nuclei showing perichromasia and contained a formation resembling an acidophilic inclusion body (Fig. 3). Electron microscopy revealed that the chromatin substance of the nuclei was attached to the nuclear membrane and showed central condensation (Fig. 4); however, no viral particles were seen. In the regional lymph node the follicles with a secondary reactive centre had increased in number, became distinctly outlined, and contained pyroninophilic lymphoblasts (centroblasts and immunoblasts) having large nuclei with pale chromatin substance (Figs 5 and 6). They were surrounded by a ring, so-called "corona", consisting of small lymphocytes. In the sinusoids there was macrophage proliferation and enhanced phagocytic activity. In the Malpighian bodies of the spleen, in the vicinity of the central arteries follicles having a large secondary reactive centre developed, whereas the width of the periarteriolar zone consisting of differentiated small lymphocytes did not change as compared to the control (Fig. 7). In the mucosa of the tonsils, follicles having a secondary reactive centre had become distinct, sharply delineated as compared to the control (Fig. 8) and their average diameter increased. The epithelium was infiltrated, in segments or diffusely, by lymphocytes and lymphoblasts. The brain, lumbar spinal cord, intervertebral ganglia and sciatic nerve (n. ischiadi-

cus) were negative. No viral particles were seen in the organs by electron microscopy.

On postvaccination day 14 no inclusion bodies were observed in the nuclei of histiocytes at the site of injection. In the lymphoid organs, mainly around the germinal centres, marked proliferation of plasma cells was noted, in comparison with the earlier state.

By postvaccination days 21 and 28 angiofibroblast tissue rich in fibroblasts had appeared at the site of injection (in the puncture channel and around it). Later on this was replaced by connective tissue consisting of mature fibrocytes and collagenic fibres. On day 28 in the lymphoid organs part of the follicles contained moderate numbers of lymphoblasts: at the same time, they were richer in blood capillaries and reticular fibres.

Discussion

In the past decades the kinetics of antibody production induced by virulent AyV, or by vaccines used in the prophylaxis of Aujeszky's disease of pigs, has essentially been elucidated (Bartha and Kojnok, 1962; Surján and Kojnok, 1965; McFerran and Dow, 1975; Tanyi et al., 1978). However, little is known about the cell-mediated immune response and its regulation.

The present experiments have revealed that part of the immunomorphological changes developing after the administration of an attenuated AyV vaccine closely correlate with the persistence of the viral antigen. In essence: the acidophilic inclusion body-like formations were observed in the histiocytes at the site of vaccination only until the viral antigen disappeared. In the lymph follicles the lymphoblasts decreased in number after the macrophage-bound antigen had been eliminated.

As to the dignostic importance of the inclusion body-like formations seen at the site of injection and the appearance of viral antigen in the tonsils, it should be noted that virulent AyV infection leads to the development of "real" intranuclear inclusion bodies (Csontos and Széky, 1966; Bergmann and Becker, 1976). With knowledge of the time of vaccination, viral antigen demonstrable in the tonsils does not impair the reliability of the IF test (Mocsári and Süveges, 1985) in the diagnosis of Aujeszky's disease.

IC developing as a result of antigen-antibody coupling during viral infections have been shown to be able to alter the pathomechanism and course of certain diseases (Oldstone and Dixon, 1971; Trautwein, 1982). On the other hand, as "positive" or "negative" signals they may play an important role in immunoregulation (Theofilopoulos and Dixon, 1979; Kölsch et al., 1980). Our results show that IC can be demonstrated as early as day 4 after administration of the attenuated virus vaccine, i.e. before detectable levels of VN anti-

bodies appear. They reach the maximum on day 7 and disappear by day 14. Parallel to this, the LST, RCF and CT reactions increase in intensity. As no deposition of IC was found in the tissues, it can be assumed that the IC bind to immunocompetent cells and act as a "positive" signal on the function of the latter.

The sensitivity of the LST induced by AyV has been studied by several authors (Ashworth et al., 1979; Alva-Valdes et al., 1983; Wittmann et al., 1976; Oirschot, 1978). Wittmann et al. (1985*a*) reported that the lymph node and spleen cells of pigs experimentally infected with virulent virus gave a positive response to an *in vitro* antigenic stimulus 4–6 days after infection. The blood lymphocytes of pigs infected in the same way react from day 6 onward (Bearan et al., 1980). In samples from animals immunized with an attenuated vaccine, the positive stimulation index appeared between days 7 and 14 (Oirschot and Gielkens, 1984). In the present experiments, in samples taken from the vaccinated pigs by day 4 the rate of blastogenesis did not reach the threshold of positivity; a positive stimulation index was first reported as late as day 7.

As shown by immunorosette formation, blood lymphocytes formed rosettes with antigen-sensitized SRBCs from day 4, whereas lymphocytes isolated from the spleen and lymph nodes from day 7 onward. A comparison with results of other tests, such as the leucocyte migration-inhibition procedure (Gutekunst, 1979) or the skin test (Smith and Mengeling, 1977), which in the naturally infected pigs are positive already on day 4 and 7, respectively, shows that the RCF test indicates sensitization of the organism as early as the latter procedures.

Our results concerning the cytotoxic capacity induced by the attenuated vaccine are different from those reported for samples of experimentally infected pigs and those immunized with an inactivated vaccine (Wittmann et al., 1985*a*; Wittmann et al., 1985*b*). With lymphocytes separated from the blood of experimentally infected pigs, Wittmann et al. (1985*a*) demonstrated antibody-dependent CT reactions from days 9–11 onward. The reactions decreased in intensity between day 14 and day 21. We assume, however, that in the early stage of the immune response to AyV an antibody-independent CT capacity may also develop.

Summarizing the *in vitro* reactions of peripheral lymphocytes observed in the present study, it can be stated that the cell-mediated immune response also plays an important role in the development of immunity in pigs vaccinated with an attenuated Aujeszky's disease vaccine.

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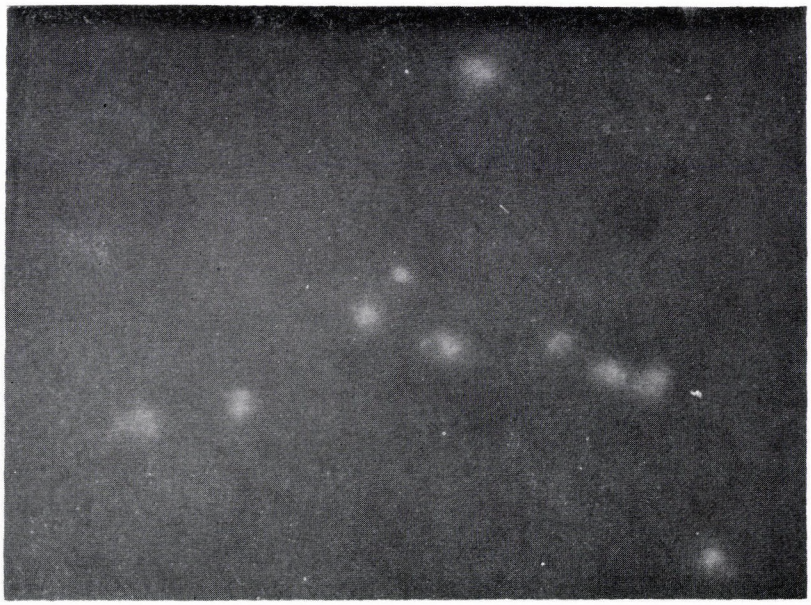


Fig. 1. Antigen-carrying macrophages in the lymph node. IF, ×300

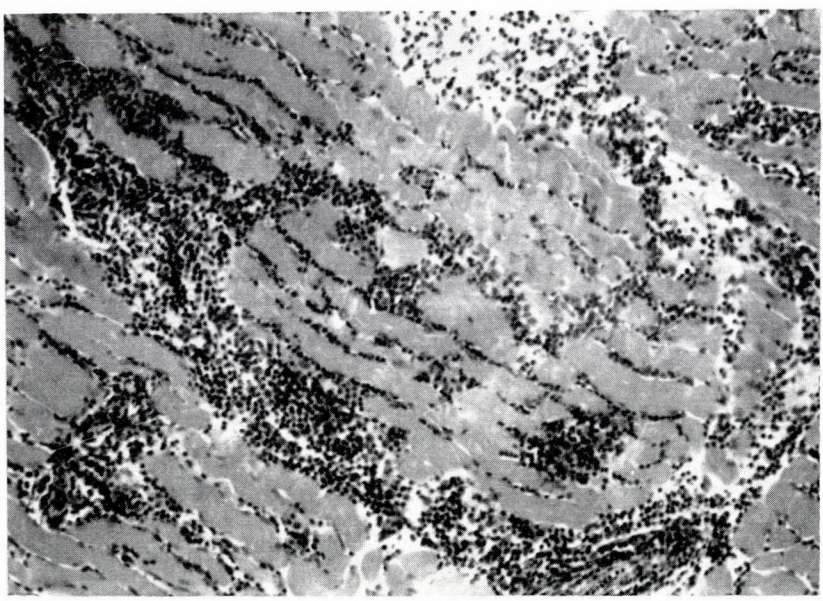


Fig. 2. Thigh muscle from a pig immunized with Aujeszky's disease vaccine. On day 4 at the site of injection there is lympho-histiocytic infiltration among the muscle fibres. Haematoxylin and eosin (H. and E.), ×63

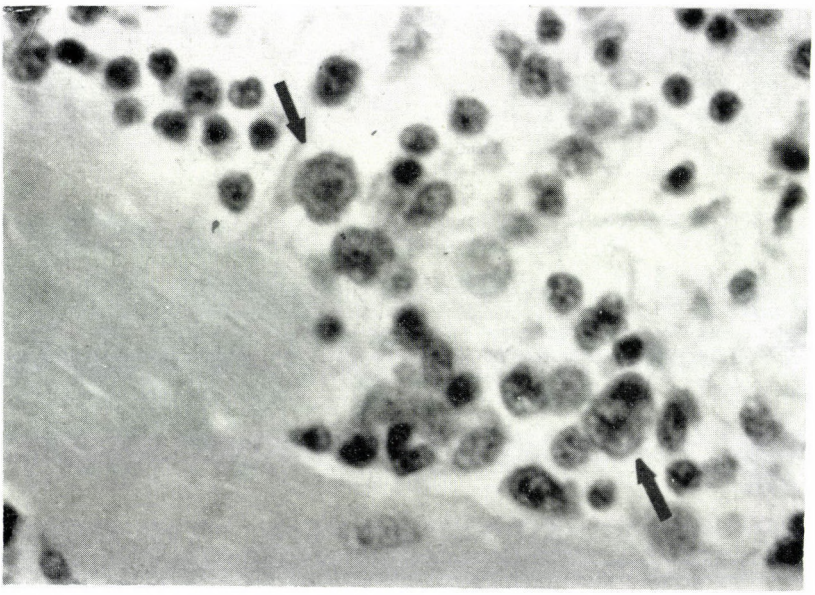


Fig. 3. Detail of Fig. 2. An inclusion body-like formation (arrow) in the nuclei of some histiocytes. H. and E., ×400

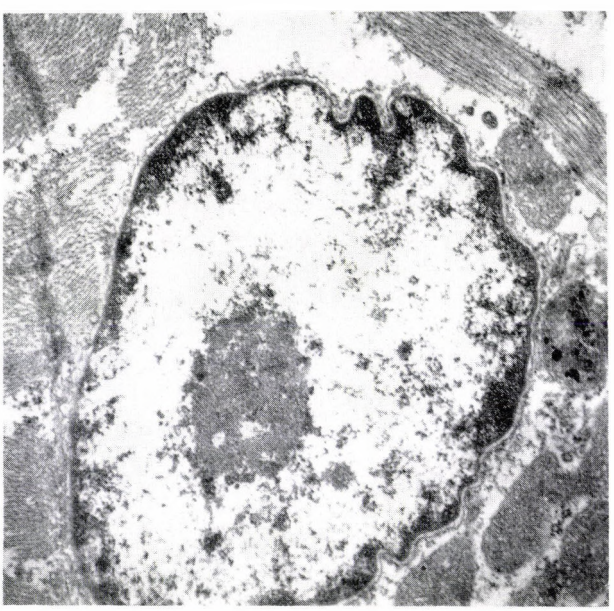
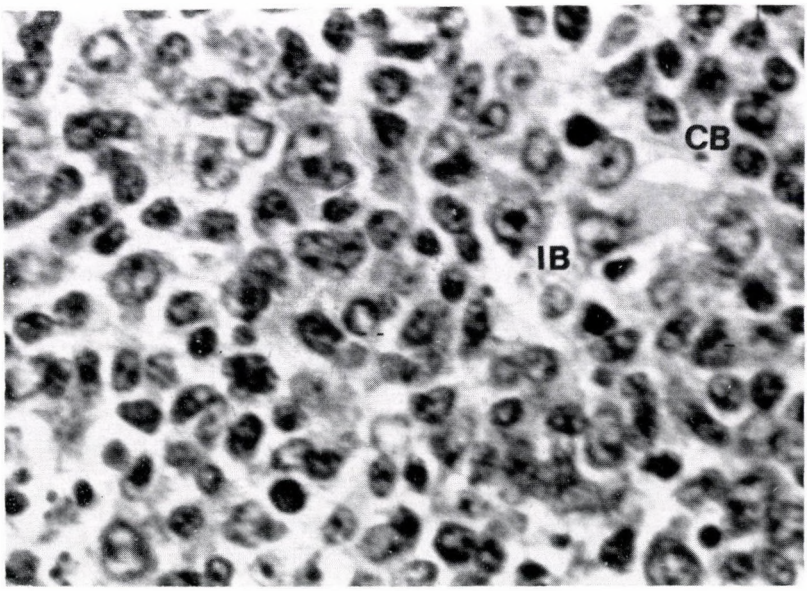
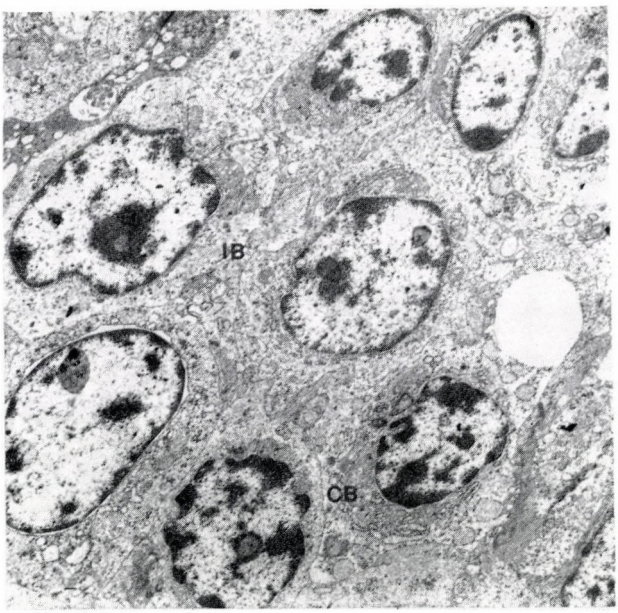


Fig. 4. Thigh muscle from a pig immunized with Aujeszky's disease vaccine. In the nuclei of some histiocytes part of the chromatin is attached to the nuclear membrane, while the remaining part is conglomerated centrally. No viral particles are seen. Electron micrograph, ×30,000





Figs 5 and 6. Popliteal lymph node from a pig immunized with Aujeszky's disease vaccine. On day 7 in the germinal centres of the cortex the nuclear chromatin of some lymphoblasts is conglomerated centrally (immunoblast, IB), while in others the chromatin substance shows irregular granular distribution (centroblast, CB). H. and E., ×400 (Fig. 5) and electron micrograph, ×6400 (Fig. 6)

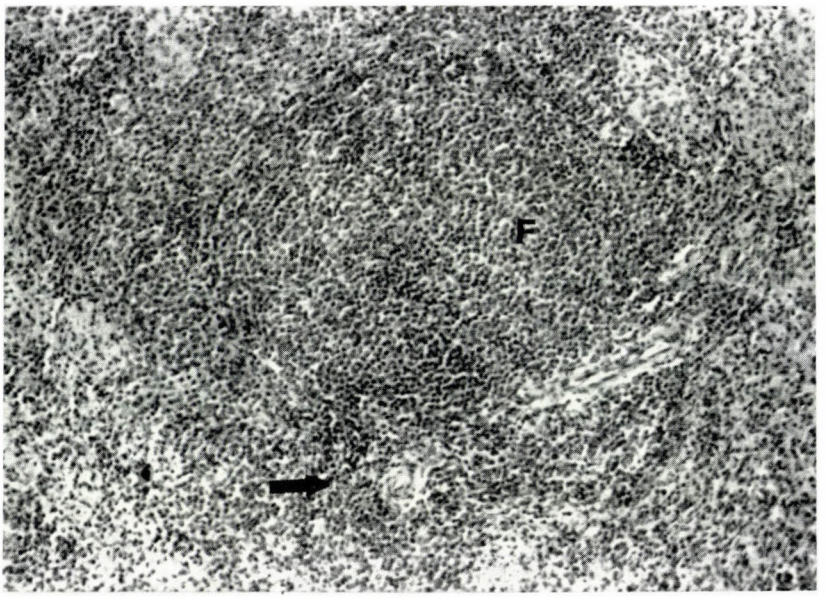


Fig. 7. Spleen from a pig immunized with Aujeszky's disease vaccine. On day 7, the follicles having a secondary reactive centre are large (F), but the periarteriolar lymphocytic zone around the a. centralis (arrow) is narrow. H. and E., ×63

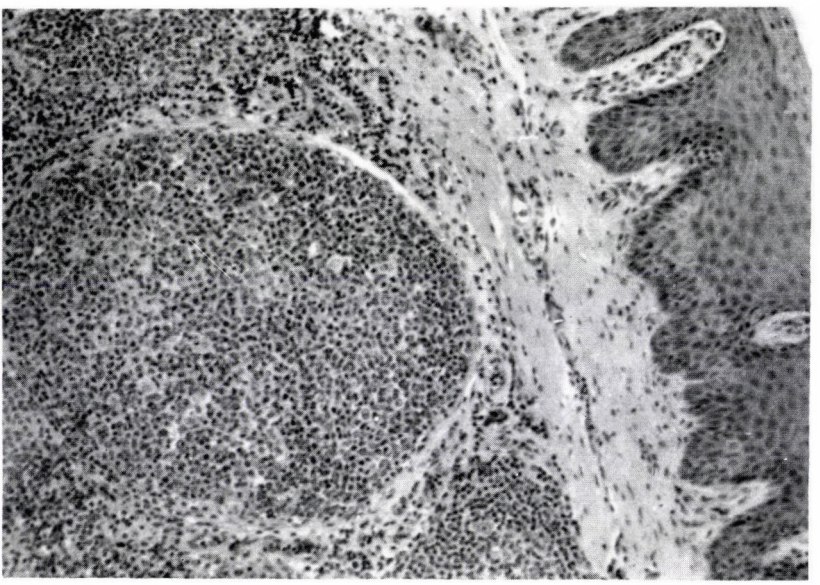


Fig. 8. Pharyngeal tonsil from a pig immunized with Aujeszky's disease vaccine. On day 7 the enlarged follicle having a secondary reactive centre is sharply delineated. H. and E., ×63

Acta Veterinaria Hungarica 36 (1–2), pp. 21–26 (1988)

A COMPLEX METHOD FOR EVALUATION OF SKIN-SENSITIZING TESTS

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A complex method is described for evaluation of skin-sensitizing tests in which erythema is also rated at the subcutis side, and total and differential cell counts are performed in the responding skin/exudate smears. The method enables a rapid, quantitative and reliable recognition and classification of the various sensitization responses including contact dermatitis.

Keywords: Skin sensitization, quantitative classification, erythema, skin/exudate smears.

Since the report of Landsteiner and Jacobs (1935) the demonstration of skin sensitization has still been performed macroscopically. The evaluation of the skin-sensitizing properties of a chemical requires the use of 40–50 guinea pigs with the most reliable methods (Buechler, 1965; Magnusson and Kligman, 1969; Marzulli and Maibach, 1977). The great number of animals, including solvent and positive controls, makes it possible to obtain various semiquantitative indices, but each with subjective elements.

Extension of erythema and degree of oedema can be quantified, but these parameters are mainly characteristic of the exudative inflammatory reactions (Gad and Dunn, 1986). Contact dermatitis is, however, classified as a delayed-sensitization response (Salvin, 1965) with predominance of the proliferative stage of inflammation.

We have therefore developed a complex method for evaluation of the various skin-sensitizing tests by rating erythema also at the subcutis side and determining total and differential cell counts in the skin/exudate smears.

Materials and methods

Animals and treatments

Eighty-four albino guinea pigs of the DPH strain (LATI, Gödöllő, Hungary) were used. The test materials were applied by epicutaneous (ec.) or intradermal (id.) route, on the scapular region of each animal, after depilation

of an area of approximately 30×60 mm. The sensitizing treatments detailed in Table I were conducted over 10 consecutive days. One week later the challenge doses were applied on the opposite flanks of the animals in the same manner.

Determination of the extension of erythema

Extension of external erythema was determined at various intervals after challenge by measuring the minimal and maximal diameters of the responding skin area. The skin was then carefully peeled from the muscles and resected under deep ether anaesthesia. The skin samples were then turned to the light with the external side and diameters of erythema visible from the inner side were taken as before (Figs 1 and 2). The size of the erythematous area was expressed in mm^2 by multiplying the two diameters.

Preparation of skin cytosmears

The skin samples in the centre of response were cut into two, and smears were prepared by pressing the fresh cut surfaces to clean slides. The dry smears were fixed in methanol for 5 min, then stained with haematoxylin and eosin. The total cell count was determined in 10 visual fields per smear with parallel performance of differential count in these fields, similarly to that of leucocytes, under a magnification of ×400.

The applicability of our method is exemplified by evaluation of the skin-sensitizing ability of Ipam 40 and DNCB with solvent controls in guinea pigs (Table I).

Results and discussion

The sensitization responses to the chemicals used were classified according to Draize's index (1959). The results are presented in Table I.

Extension of erythema

The erythema at the subcutis side appeared larger than the external one in each case since scoring was free from disturbances by oedema, thickening of epidermis and growing hair. The difference among weak, moderate and strong responses can be demonstrated by figures (Table II). The difference between the external and internal erythema scores is particularly great in animals treated with Ipam 40. Supposedly, the degree of the sensitizing response could be stronger since erythema could be recognized at the subcutis side when no external response was noted.

Table I

Test materials, animals used in sensitizing tests and classification of the sensitization responses according to Draize (1959)

Test material	Concentration, g/v %	Volume, ml	Vehicle	Route	No. of animals treated	Judging of sensitization responses on the basis of Draize's index
Distilled water	—	0.5	—	ec.	12	Negative
Ethanol, dehydrated		0.5	—	ec.	12	Negative
DNCB	0.1	0.5	ethanol	ec.	12	Weak
DNCB	1.0	0.5	ethanol	ec.	12	Moderate
DNCB	0.1	0.1	sunflower oil	id.	24	Strong
Ipam 40	20.0	0.5	distilled water	ec.	12	Weak

DNCB = dinitro-chloro-benzene; Ipam 40 = a pesticide containing 40% ammonium-N-methyldithiocarbamate; ec. = epicutaneous (topical) application; id. = intradermal injection; DNCB and Ipam 40 were applied at subirritative dosages

Table II

Extension of the erythematous areas at the external and at the subcutis (internal) side at various intervals after challenge

	Test material/Route		24 h	48 h	72 h	96 h	Judging of sensitization responses on the basis of Draize's index
			Ext./Int.				
	Distilled warer	ec.	0/0	0/0	0/0	0/0	Negative
	Ethanol	ec.	10/50*	0/30	30/50*	30/30*	Negative
Sub-irritative	0.1% DNCB	ec.	71/130	50/150	32/60	0/0	Weak
	1.0% DNCB	ec.	110/180	95/149	86/145	102/100	Moderate
	0.1% DNCB	id.	158/202	400/495	188/592	128/543	Strong
	20.0% Ipam 40	ec.	130/160	68/247	80/247	0/118	Weak

For explanation, see footnote to Table I

* The unusual erythema caused by ethanol was probably due to contamination/impurities of ethanol, a fact supporting the need of using solvent controls

Total cell count

Based on our previous experiments, the cell count is directly related with the severity of inflammation.

In our example (Table III) the difference in total cell count between the weak and moderate skin responses is conspicuous but it varies disproportionally from the moderate to strong reactions. The fact is due to a severe oedema produced by intracutaneously administered DNCB, which resulted in a decrease in total cell count (Fig. 3). The subsequent cytosmears from this treatment were also richer in cells.

Table III

Total cell counts in skin/exudate smears per visual field (×400) at various intervals after challenge

	Test material/Route		24 h	48 h	72 h	96 h	Judging of sensitization responses on the basis of Draize's index
			(mean ± SD)				
	Distilled water	ec.	0.93 ± 0.83	1.23 ± 1.41	0.93 ± 1.01	1.03 ± 1.23	Negative
	Ethanol	ec.	2.00 ± 1.82	1.50 ± 1.22	1.33 ± 1.21	1.87 ± 2.73	Negative
Sub-irritative	0.1% DNCB	ec.	4.78 ± 4.61	5.49 ± 6.71	2.90 ± 3.06	2.37 ± 2.47	Weak
	1.0% DNCB	ec.	69.34 ± 74.92	60.20 ± 55.71	38.46 ± 29.36	25.14 ± 27.93	Moderate
	0.1% DNCB	id.	16.93 ± 10.92	36.47 ± 20.51	70.17 ± 49.29	10.53 ± 14.94	Strong
	20.0% Ipam 40	ec.	6.17 ± 6.80	9.47 ± 9.49	12.60 ± 18.46	8.97 ± 14.81	Weak

For explanation, see footnote to Table I

Table IV

Differential cell counts (per cent) in the skin/exudate smears at various intervals after challenge

	Test material/Route		24 h				48 h				72 h				96 h				Judging of sensitization responses on the basis of Draize's index
			SE	EO	LY	MA	SE	EO	LY	MA	SE	EO	LY	MA	SE	EO	LY	MA	
	Distilled water	ec.	18	0	56	26	18	1	72	9	4	0	77	19	6	0	66	28	Negative
	Ethanol	ec.	20	0	67	13	18	0	67	15	15	2	74	9	10	4	71	15	Negative
Sub-irritative	0.1% DNCB	ec.	11	2	64	23	10	1	59	30	12	3	61	24	6	1	71	22	Weak
	1.0% DNCB	ec.	65	1	5	29	44	1	26	29	8	1	46	45	3	1	82	14	Moderate
	0.1% DNCB	id.	23	6	48	23	30	2	35	33	25	3	39	33	3	1	41	55	Strong
	20.0% Ipam 40	ec.	14	1	33	52	2	0	36	62	4	1	52	43	9	1	55	35	Weak

For explanation, see footnote to Table I
SE: segmented neutrophilic leucocyte; EO: eosinophilic leucocyte; LY: lymphocyte; MA: macrophage

The cell count in response to Ipam 40 was slightly higher compared to the low dosage of DNCB and the smears from the solvent controls were very poor in cells.

Differential count

Contrary to the previous methods, the qualitative composition of the cytosmears (Table IV) can indicate the proliferative character of inflammation and represent the complete sphere of cell types occurring in contact dermatitis (Benaceraff and Green, 1969; David and David, 1972; Uhr, 1966).

The sensitization response is the most demonstrative in the exudate from animals treated ec. with 1% DNCB. At 24 h after challenge, the smears contained segments in high proportion, characteristic of the vascular stage of inflammation. They were gradually replaced by mononuclear elements representing the proliferative stage. Within this stage the macrophages and lymphocytes increased in number for 72 and 96 h, respectively.

The pattern of skin sensitization in the case of DNCB corresponds to the general pattern of the delayed-type sensitization responses: invasion of polymorphonuclears for 4–8 h, followed by mononuclear elements: macrophages, then lymphocytes. However, in the case of Ipam 40 the skin samples already contained macrophages in a high proportion 24 h after challenge.

In summary, the method presented here offers several advantages over the traditional macroscopic evaluation. Due to its complexity, it enables a rapid, quantitative and reliable judging of the sensitization responses.

Determination of erythema at the subcutis side together with total and differential counts in the responding skin/exudate smears provides objective and reproducible data. These parameters are at the same time good indices of the character of the inflammatory responses, including contact dermatitis. Early presence of macrophages in high proportion in the skin is particularly important from this respect.

Based on these data and our three-year experience we suppose that this method can serve as a new approach to a more reliable evaluation system of the various skin-sensitizing tests.

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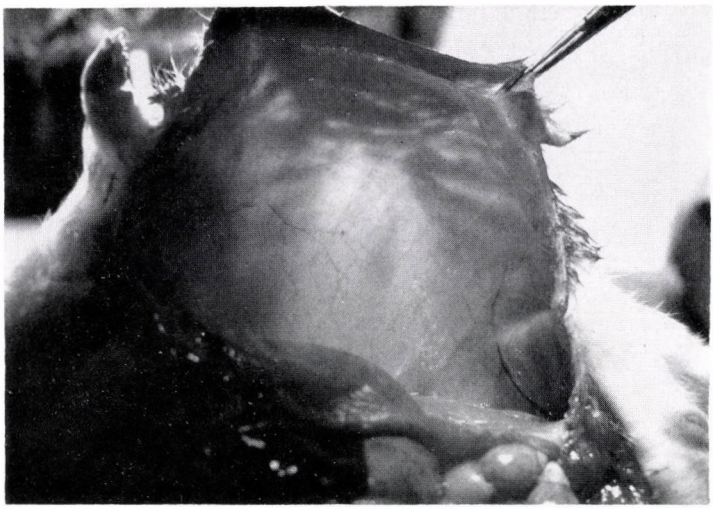
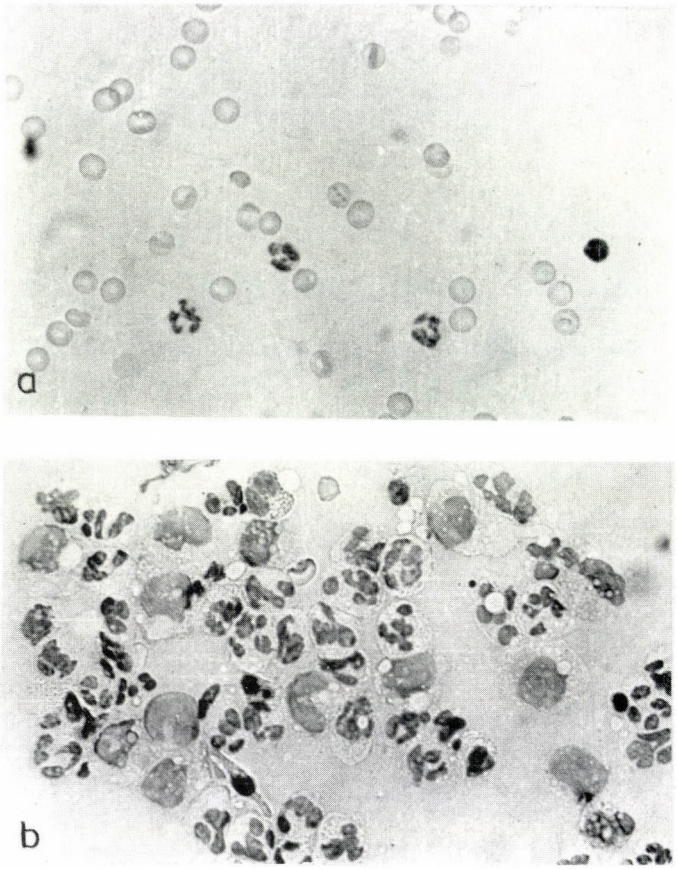


Fig. 1. Erythema at the subcutis side from a guinea pig treated ec. with ethanol, 24 h after challenge



Fig. 2. Erythema at the subcutis side from a guinea pig treated ec. with 1.0% DNCB, 24 h after challenge


a
b

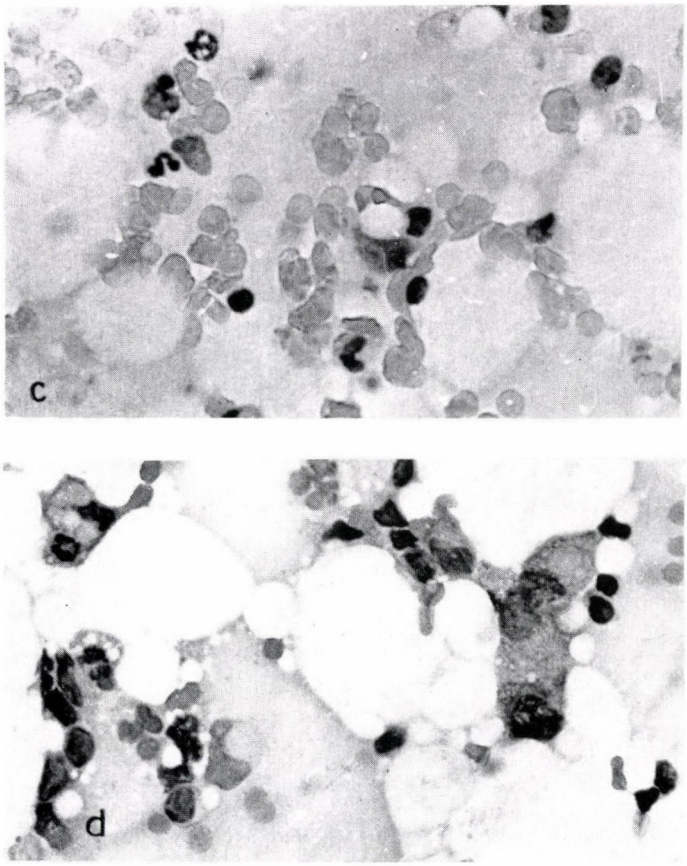


Fig. 3. Skin-cytosmears from guinea pigs (a) 24 h after ec. challenge with ethanol; (b) 24 h after ec. challenge with 1.0% DNCB; (c) 24 h after id. challenge with 0.1% DNCB; (d) 72 h after id. challenge with 0.1% DNCB. Haematoxylin-eosin, ×750

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DIACETYL REDUCTASE ACTIVITY IN THE EXTRACTS OF *FASCIOLA GIGANTICA*

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Diacetyl reductase catalyzed the reduction of diacetyl, using either NADPH or NADH as hydrogen donors. The enzyme had a higher affinity for NADPH than for NADH. It showed maximal activity at pH 6.0. At pH 6.0 and 0.016 mM NADPH, the apparent K_m for diacetyl has been estimated to be 0.092 mM. The enzyme does not require metallic ions for activity. Thiol blocking agents showed little effect, indicating that the enzyme does not possess —SH groups at the active site. The characteristics of diacetyl reductase from *Fasciola gigantica* are similar to that of the same enzyme from beef liver on the basis of pH, K_m value and the effect of metallic ions on its activity.

Keywords: Diacetyl reductase, activity, characteristics, extract, *Fasciola gigantica.*

Diacetyl reductase (acetoin: NAD^+ oxidoreductase, EC 1.1.1.5), which brings about the enzymatic transformation of diacetyl to acetoin coupled to NADPH or NADH oxidation, has been reported from bacteria (Branen and Keenan, 1970; Silber et al., 1974; Kaneko et al., 1986), mammalian tissues (Gabriel et al., 1971; Burgos and Martin, 1972; Provecho et al., 1984) and bird tissues (Burgos et al., 1974; Diez et al., 1974). An up-to-date scanning of the literature, however, shows that this enzyme has not been studied so far in any of the parasitic helminths. Therefore, it appeared justifiable to investigate the presence, if any, of diacetyl reductase and its characteristics from *Fasciola gigantica*, a trematode having widespread occurrence in the bile ducts of buffaloes *(Bubalus bubalis)*, which are highly prized domestic animals in India for milk and meat.

This communication reports the presence and certain characteristics of diacetyl reductase in this trematode.

Materials and methods

Adult *F. gigantica* specimens were collected from the bile ducts of buffaloes slaughtered at the local abattoir and washed several times in cold normal saline (0.72 w/v, NaCl). Only flukes that had disgorged their caecal contents completely were selected for enzyme purification. The subsequent operations were carried out at 0–4 °C. The biochemicals used in the present study were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

The enzyme activity was measured by following the rate of change of absorbance at 340 nm (1 cm light path) with Spectronic-21 at 30 °C. The reaction mixture (1.5 ml) contained 50 mM phosphate buffer (pH 6.1), 0.016 mM NADPH, 5 mM diacetyl, and the enzyme. One unit of enzyme was defined as the amount which oxidized 1 μmol NADPH per min.

The worms were homogenized in a blender for 2 to 3 min in 3 volumes of 0.1 M phosphate buffer (pH 7.0) containing 5 mM 2-mercaptoethanol. The homogenate was centrifuged at 22,000 *g* for 30 min at 4 °C (Sorval Model RC_2-8) and the supernatant fluid was our crude extract. Maximum measurable activity for diacetyl reductase was in the fraction precipitating at 4 °C with 60% saturated ammonium sulphate after removal of the precipitate formed with 30% saturation. The precipitate was dissolved in a minimal volume of 0.1 M phosphate buffer (pH 6.1) and dialyzed overnight in cold against 100 volumes of the same buffer. This enzyme preparation was used for the characterization studies. All observations were made in three or four extracts.

For determination of pH optima, a series of 50 mM phosphate buffer (pH 5.5 to 8.0) and a series of 50 mM Tris-HCl buffers (pH 8.5 to 9.5) were used under the standard assay conditions.

Using the above-mentioned assay mixture with different concentrations of diacetyl, its K_m value was determined by the Lineweaver-Burk double reciprocal plot.

For cofactor specificity the same concentrations (0.016 mM) of NADPH or NADH were used in the above assay mixture.

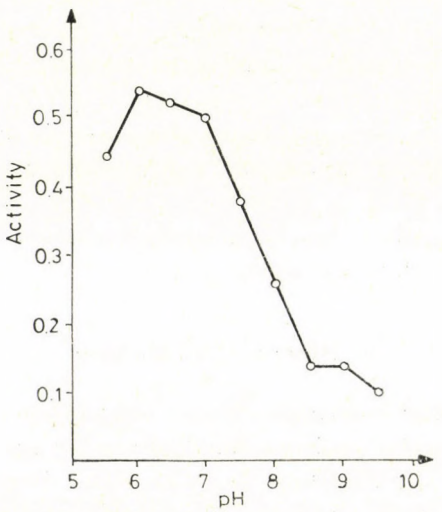


Fig. 1. Effect of pH values on the enzyme activity. The assay mixture (1.5 ml) contained 50 mM phosphate buffer (pH 5.5 to 8.0) or Tris-HCl (pH 8.5 to 9.5), 0.016 mM NADPH, 5 mM diacetyl, and the enzyme

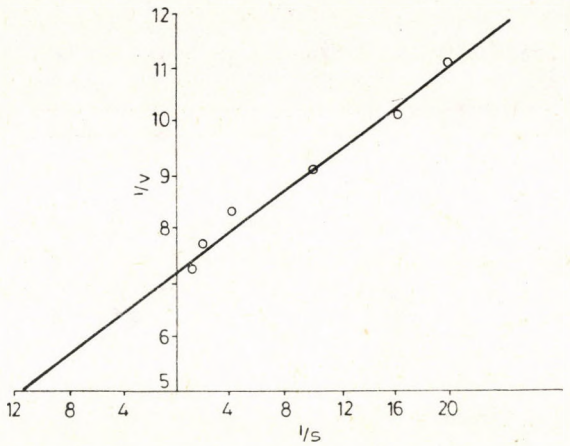


Fig. 2. Lineweaver-Burk plot for *Fasciola gigantica* diacetyl reductase by varying diacetyl concentrations. The assay mixture contained 50 mM phosphate buffer (pH 6.1), 0.016 mM NADPH, the enzyme, and 1 mM, 0.5 mM, 0.25 mM, 0.1 mM, 0.0625 mM, or 0.05 mM diacetyl

To observe the effect of thiol blocking agents and metal chelators, the enzyme was incubated with 1 mM compound for 5 min at room temperature and then assayed for enzyme activity.

Results

Diacetyl reductase from *F. gigantica* showed maximum activity at pH 6.0 (Fig. 1).

The K_m value for diacetyl was 0.092 mM (Fig. 2).

Table I

Effect of additives on diacetyl reductase activity

Additive	Concentration (mM)	% activation (+) or inhibition (—)
Metal chelators		
EDTA	1	+10
Phenanthroline	1	— 3.74
Na-pyrophosphate	1	no effect
Cysteine	1	+ 3.3
Thiol blocking agents		
Iodoacetic acid	1	+10.2
Iodoacetamide	1	+15.7
N-ethylmaleimide	1	— 4.5

The above values are the means of the four extracts

Table II

Data on optimum pH and diacetyl K_m values of diacetyl reductase from different tissues

Tissue	pH optima	K_m (mM)	Reference
*Fasciola gigantica**	6.0	0.092	Present paper
Beef liver	6.1	0.0398 0.067	Martin and Burgos (1972) and Diez et al. (1974)
Pigeon liver	5.6–6.0	3.1	Diez et al. (1974) and Burgos et al. (1974)
Rat liver	—	48	Gabriel et al. (1971)
Lactobacillus casei	4.5	2.1	Branen and Keenan (1970)
Aerobacter aerogenes	5.8	1.2	Larsen and Stormer (1973) and Johansen et al. (1973)
Escherichia coli	6.0	4.4	Silber et al. (1974)

* Averages based on three extracts

Cofactor specificity studies revealed that NADPH gave better response than NADH, as the enzyme activity was 0.24 units/ml with NADPH and 0.18 units/ml with NADH.

As shown in Table I, the effect of thiol blocking agents (iodoacetic acid, iodoacetamide, N-ethylmaleimide) and metal chelators (EDTA, phenanthroline, Na-pyrophosphate, and cysteine) was slight and insignificant.

Discussion

The results reported above show that diacetyl reductase preparations from *F. gigantica* are able to catalyze the reduction of diacetyl, coupled to the oxidation of either NADPH or NADH. The enzyme had a higher affinity for NADPH than for NADH, similarly to the enzyme from beef liver (Provecho et al., 1984).

The pH optimum (6.0) for the experimental enzyme is close to that for beef liver (Martin and Burgos, 1972), pigeon liver (Diez et al., 1974), *Aerobacter aerogenes* (Larsen and Stormer, 1973), and *Escherichia coli* (Silber et al., 1974). These values (Table I) indicate that the reduction of diacetyl is maximally favoured at around pH 6.0.

The low K_m value of diacetyl in the present enzyme is similar to that of beef liver reported by Martin and Burgos (1972) and Diez et al. (1974), indicating that in both cases diacetyl appears to be a favourable substrate. The K_m values of diacetyl for diacetyl reductases from other sources, reported by various workers, show very wide variation (Table II).

The lack of any substantial effect of metal chelators on diacetyl reductase from *F. gigantica* shows that the enzyme does not require metallic ions for its activity (Table II). Non-requirement of metal ions for the activity of diacetyl reductases has also been reported from beef liver (Burgos and Martin, 1972), pigeon liver (Diez et al., 1974), and *Streptococcus diacetylactis* (Seitz et al., 1963).

When tested with thiol blocking agents (Table II), the test enzyme showed little effect, indicating that it does not possess —SH groups at the active site. This characteristic does not appear to have been studied so far for this enzyme from other sources.

It is concluded that the presence of diacetyl reductase in *F. gigantica* has been recorded for the first time. The characteristics of this enzyme show it to be similar to that found in beef liver on the basis of pH, K_m value, higher affinity for NADPH, and lack of effect of metallic ions on its activity.

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CHLAMYDIA-INDUCED ABORTION IN A HORSE

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In two equine fetuses (twins) aborted in the 7th month of pregnancy, chlamydiae were demonstrated by light-microscopic examination of smears made from the stomach contents and fetal membranes and by transmission electron microscopy of the livers. The aetiological involvement of other abortifacient infective agents was ruled oud

Necropsy of the fetuses revealed icterus, enlargement of the spleen, liver ant. certain lymph nodes, scattered petechial or, occasionally, extensive haemorrhages in. the subcutaneous tissue, skeletal muscles, under the serous and in the mucous membranes, and in the placentae.

Histologically there was serous hepatitis characterized by activation and, in some places, focal proliferation of cells of the mononuclear phagocyte system (MPS) and fetal extramedullary haemocytopoiesis, vacuolar degeneration of parenchymal cells in the centrolobular areas, lympho-histiocytic infiltration of the periportal tissues, and signs of cholestasis. In addition, in the spleen and lymph nodes hyperaemia and proliferation of MPS and haemocytopoietic cells, among the myocardial fibres focal lymphohistiocytic infiltrations, in the lungs hyperaemia, in the kidneys bilirubin nephrosis, and in the placentae haemorrhages were seen.

By transmission electron microscopy, chlamydial developmental stages were identified in vacuoles situated in the cytoplasm of MPS cells in the liver.

Keywords: Chlamydia, abortion, horse, gross pathology, histopathology, electron microscopy.

Little is known about chlamydial diseases of the horse. Earlier, chlamydiae had been incriminated as potential aetiological agents of recurring iridocyclochoroiditis (so-called moon-blindness) of adult horses; however, attempts at isolating them from affected horses consistently failed (Storz, 1971). Experimental infection with a chlamydia strain isolated from caprine pneumonia resulted in pneumonia in horses (Saito, 1954). Others (Popovici and Hiastru, 1968) isolated chlamydiae from the blood of a young foal diseased in pneumonia; however, they failed to report on any infection experiment performed with this strain. Blanco Loizelier (1968; cited by Pienaar and Schutte, 1975) demonstrated the pathogen in the brain of adult horses and investigated its possible role in the aetiology of the so-called hepatocerebral syndrome.

Following experimental infection with chlamydiae, Erasmus (personal communication, 1973; cited by Pienaar and Schutte, 1975) observed abortion, whereas on the basis of similar experiments Ostrovski (1981) reported that, as compared to the control, experimentally infected mares delivered foals of lower viability and body mass at full term.

On one occasion each, in the Republic of South Africa and in Spain chlamydiae were brought into aetiological connection with spontaneous abortion in mares (Bosman et al., personal communication, 1973; cited by Pienaar and Schutte, 1975; Blanco Loizelier et al., 1976).

Case report

Two equine fetuses (twins) and placentae aborted in the 7th month of pregnancy were submitted for diagnostic examination. Case history indicated no other abortions or apparent clinical disease among the mares. At necropsy of the fetuses, icterus, considerable enlargement of the spleen, liver and certain lymph nodes, and scattered petechial or, rarely, extensive haemorrhages in the subcutaneous connective tissue, skeletal muscle, under the various serous membranes, in the mucous membranes and placentae were seen.

Histologically there was serous hepatitis in both fetuses (Fig. 1), characterized by activation and, in places, focal proliferation of cells of the mononuclear phagocyte system (MPS) and those of fetal extramedullary haemocytopoiesis, vacuolar degeneration of hepatocytes in the centrolobular areas, pronounced lympho-histiocytic infiltration in the periportal tissues, and signs of cholestasis. The spleen and the lymph nodes examined (the portal and peribronchial lymph nodes) exhibited hyperaemia and proliferation of the MPS cells and of cells participating in haemocytopoiesis (Fig. 2). Among the myocardial fibres there were, in places, focal lympho-histiocytic infiltrations (Fig. 3), whereas in the lungs hyperaemia and, here and there, detachment of the alveolar epithelium, in the kidneys bilirubin nephrosis, and in the placentae circumscribed haemorrhages and oedema were observed.

In smears prepared from the fetal stomach contents, surface of the gastric mucosa and from the fetal membranes, and stained according to Stamp, large numbers of bright red formations (chlamydiae) were seen, mainly intracellularly in the cytoplasm of detached epithelial cells but freely as well.

Transmission electron microscopy revealed that chlamydiae were located in the MPS cells, or in the degenerated remnants thereof, of the hepatic sinusoids, and were surrounded by the cell membrane. In these cells all the three developmental stages of chlamydiae were found, arranged in groups: namely, the so-called initial bodies (forms 0.5 to 0.7 μm in diameter, bound by double membrane and filled with a cytoplasm of low electron-density), the intermediate bodies 0.4 to 0.6 μm in diameter, showing electron-dense cytoplasmic conglomerations, and the so-called elementary bodies 0.3 to 0.4 μm in diameter, filled with a mainly electron-dense, conglomerated cytoplasm (Fig. 4).

Bacteriological examination of the fetal stomach contents, liver, lungs and splcen, carried out parallel in the presence of O_2 and CO_2, was consistently

negative, similarly to virological examination of the spleen, lungs, peribronchial and mesenteric lymph nodes for the presence of rhinopneumonitis and other viruses.

For lack of test samples, serological examination of the aborted and other mares' blood for chlamydial antibodies was not possible.

Discussion

Based upon the light-microscopic examination of smears and impression preparations and the histopathological and electron-microscopic examination of certain organs, the aetiological involvement of chlamydiae in the natural case of equine abortion observed by us seems highly probable, even without having isolated the pathogen and demonstrated seroconversion. Our observations, along with the few pertinent data available in the literature (Bosman et al., personal communication, 1973, cited by Pienaar and Schutte, 1975; Blanco Loizelier et al., 1976), suggest that, although rather infrequently, a fetopathic effect of chlamydiae should be reckoned with also in the horse. At the same time, certain authors (Studdert, 1969) reported considerable seroconversion in horses in connection with chlamydial infection. Blanco Loizelier et al. (1976) reported equine abortion accompanied by fever and polyarthritis. Most of the abortions occurred in the 8th month of pregnancy. In the present case, the case history indicated no clinically apparent disease among mares, and no abortions other than the one reported occurred in the given breeding season.

The gross and histopathological lesions seen in the fetal organs were similar to those found in ovine and bovine fetuses aborted due to chlamydial infection (Shewen, 1980; Glávits et al., 1982), the only difference being that in horses placentitis was less severe than in the other two species. In addition to the placentae and the fetal stomach contents, chlamydiae were present in the MPS cells as well, indicating that in the fetuses chlamydiaemia had developed. In the cytoplasm of these cells there were signs indicative of chlamydial growth.

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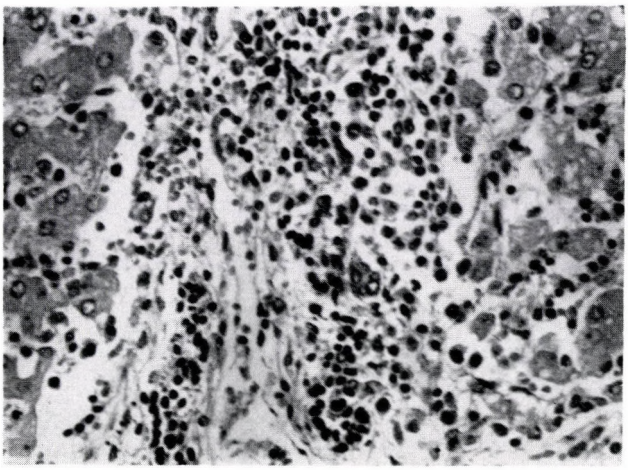


Fig. 1. Pronounced lympho-histiocytic infiltration in the periportal areas of the liver. Haematoxylin and eosin (H-E.), ×160

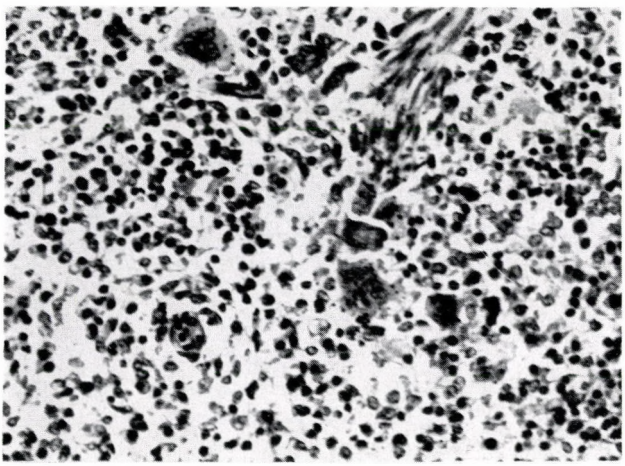


Fig. 2. Proliferation of cells participating in extramedullary haemocytopoiesis, including giant cells (megakaryocytes), in the red pulp of the spleen. H-E., ×160

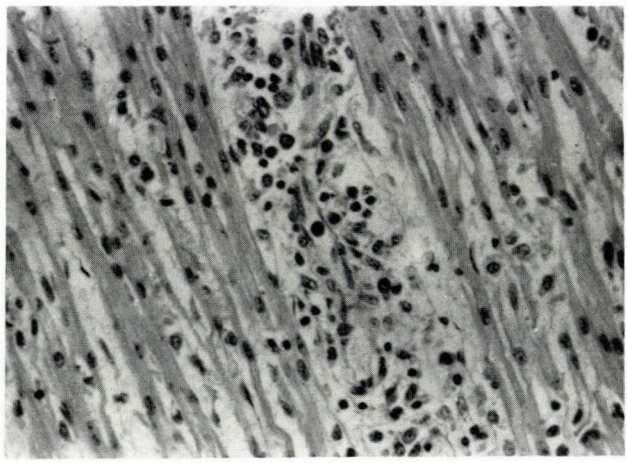


Fig. 3. Lympho-histiocytic infiltration among the myocardial fibres. H-E., ×160

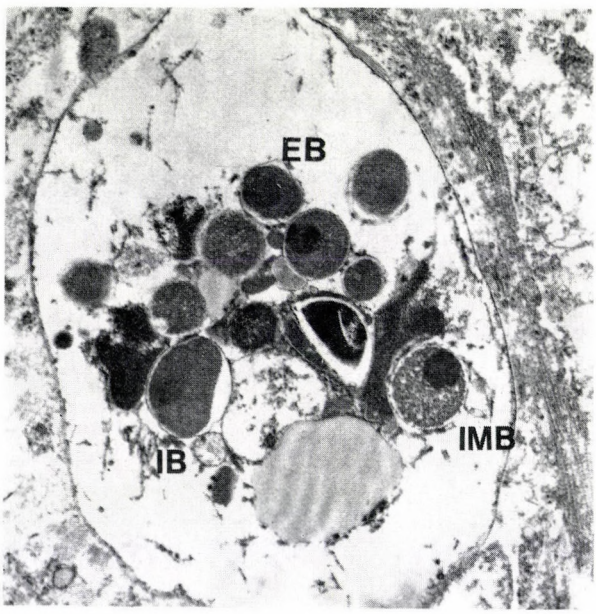


Fig. 4. Developmental stages of chlamydiae (IB = initial bodies, IMB = intermediate bodies, EB = elementary bodies) in remnants of some MPS cells, in a membrane-bound cavity, in the liver. Electron micrograph, ×14,300

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EFFECT OF TRICHOTHECENE MYCOTOXINS (SATRATOXIN H AND T-2 TOXIN) ON THE LYMPHOID ORGANS OF MICE

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Changes induced by two trichothecene mycotoxins, satratoxin H and T-2 toxin, in the lymphoid organs (spleen, thymus, prepatellar and mesenteric lymph nodes) of 120 mice were examined by morphological (gross pathological, light and electron microscopic) methods. Different doses of the mycotoxins were administered intraperitoneally, on one occasion to groups of 10 mice each.

The two mycotoxins, which are similar in chemical structure, were found to produce similar lesions in the lymphoid organs. In the killed mice as well as in those that had died after mycotoxin administration there was lymphocyte depletion and necrosis in both the B-cell-dependent and T-cell-dependent regions of the lymphoid organs. The severity of the changes was dose dependent and the lesions could be classified into one of grades I–IV on the basis of the histological findings. By comparing the LD_{50} values obtained for the two trichothecene mycotoxins, satratoxin H proved to be about five times as toxic as T-2 toxin.

Keywords: Trichothecene, mycotoxin, satratoxin H, T-2 toxin, mouse, morphology, lymphoid organs.

Cattle, sheep and horses affected with stachybotryotoxicosis exhibit ulcerative inflammation of the digestive tract mucosa, general haemorrhagic diathesis, reduced number of circulating leucocytes, and secondary infections (Dankó, 1972; Dankó and Szerafin, 1976). Dankó and Krasznai (1976) observed decreased heterohaemagglutinin titres and a reduction in the number of immunorosette-forming cells in mice, absence of cell-mediated immune response in guinea-pigs, and severe lymphocyte depletion in the B-cell-dependent and T-cell-dependent regions of the lymph nodes in sheep intraperitoneally injected with culture extracts of *Stachybotrys atra*. Hajtós et al. (1983) found that, through their immunosuppressive effect, stachybotryotoxins predispose sheep to bacterial complications caused by *Pasteurella haemolytica*.

In chickens affected by T-2 toxicosis, also an ulcerative inflammation of the digestive tract mucosa and atrophy of the lymphoid organs (lymphocyte depletion and necrosis) were found (Bitay et al., 1979, 1981). Mice diseased in T-2 toxicosis showed increased susceptibility to herpesvirus (Friend et al., 1983) and *Listeria monocytogenes* infections (Corrier and Ziprin, 1986). Studying the effect exerted by T-2 toxin on piglets, Rafai and Tuboly (1982) observed a pronounced suppression of the cell-mediated and humoral immune response both *in vitro* and *in vivo*. Friend et al. (1983) distinguished four grades in the severity of regressive lesions produced by T-2 toxin in the thymus of mice

(lymphocyte depletion, lymphocyte necrosis, indistinct demarcation of the cortex and medulla).

The purpose of the present work was to study and compare the pathological changes produced in the lymphoid organs of mice by two different, but chemically similar, trichothecene mycotoxins, satratoxin H (produced by *Stachybotrys atra*) and T-2 toxin (produced by *Fusarium sporotrichioides*).

Materials and methods

Mice. One hundred and twenty male, CFLP Swiss albino mice of 20–24 g body mass were used. Further two groups of 10 mice each served as untreated control and control treated with vehicle only.

Mycotoxins. T-2 toxin was produced in our laboratory, using *Fusarium tricinctum* syn. *sporotrichioides* (Bata et al., 1983) and was identified and quantitated by the capillary chromatographic method described earlier (Ványi et al., 1982; Bata et al., 1983). Satratoxin H was a kind gift from Árpád Bata (Department of Biochemistry and Food Technology, Budapest Technical University). Dimethyl sulphoxide (DMSO) was used as solvent.

Treatment. The mice were treated intraperitoneally as shown in Table I. The mice that died after treatment and the surviving animals killed 48 h after treatment were necropsied and examined for gross pathological lesions. For histopathological examination the spleen, thymus and the prepatellar and mesenteric lymph nodes were fixed in 5% formalin (pH 7.2) buffered with NaH_2PO_4 and NaOH. Frozen and paraffin-embedded sections were prepared. The sections were stained with P. Mayer's acid haemalaun and eosin.

Electron microscopy. Details from the spleen and mesenteric lymph node of two mice treated with satratoxin H (1.75 mg/kg body mass) and of two other mice treated with T-2 toxin (4 mg/kg body mass) and subsequently killed by bleeding were cut into fragments of appropriate size and fixed in 2.5% glutaraldehyde, then in 1% osmium tetroxide buffered with sodium cacodylate. The details to be examined by electron microscopy had been selected on the basis of the light-microscopic findings. After embedding in Durcupan resin, sections were cut with an ultramicrotome and examined in an electron microscope.

Results

The LD_{50} of satratoxin was found to be 1.51 mg/kg body mass.

Table I shows the number of deaths that occurred in the groups treated with satratoxin H or T-2 toxin. Most deaths took place between the 8th and 24th hour after treatment.

Table I

Design of the experiment and mortality among mice

Mycotoxin	Satratoxin H									T-2			Control	
													Treated with vehicle only	Untreated
Dose (mg/kg body mass) ip.	1.0	1.15	1.30	1.45	1.60	1.75	1.90	2.05	2.20	2.0	4.0	8.0		
Number of deaths/group*	—	5	2	4	5	10	7	8	10	3	4	9	—	—

* Each group consisted of 10 mice

Table II

Mycotoxin-induced changes in the lymphoid organs

The cortex and medulla of the thymic lobes	are distinct	slight lymphocyte depletion (involving maximum 40% of the lymphocyte population)	I
		pronounced lymphocyte depletion (40–80%) but only slight lymphocyte necrosis	II
The B-cell-dependent and T-cell-dependent zones of the spleen and lymph nodes	are not distinct	severe lymphocyte depletion (above 80%) and marked lymphocyte necrosis	III
		very severe lymphocyte depletion (95–100%) and necrosis extending to the reticular framework	IV

At necropsy, mice that had died of either mycotoxin exhibited hyperaemia in the spleen, liver, kidney and lungs. The mesenteric blood vessels were dilated and filled with blood and, occasionally, a serous exudate had accumulated in the pericardium. In one mouse of both groups the stomach and small-intestinal mucosa was slightly swollen and hyperaemic and exhibited, here and there, petechiae. Rarely there were superficial erosions of the mucous membrane in the fundus of the stomach. The control mice and the mice killed 48 h after mycotoxin treatment did not exhibit appreciable gross pathological lesions at necropsy.

By light and electron microscopic examination, similar histopathological changes were found in the lymphoid organs of mice treated with either of the two mycotoxins. The only differences observed were in the severity of the changes.

As compared to the control (Fig. 1) there was a striking reduction in the number of lymphocytes (lymphocyte depletion) in the cortex of the thymus,

in the germinal centres and paracortical zones of the lymph nodes (in regions of the B-cell-dependent and T-cell-dependent lymphocyte populations), in the germinal centres of the Malpighian bodies (B-cell-dependent regions) and in the periarteriolar lymphocyte zones (T-cell-dependent regions) of the spleen (Figs 2, 3 and 4). As a result, the above-mentioned zones had become less distinctly demarcated from the surrounding regions. Most of the lymphocytes that had remained in these regions showed signs of degeneration or necrosis (karyopycnosis, karyorhexis). The cell membrane and nuclear membrane had undergone lytic changes, the structure of the cytoplasm disintegrated, the cell organelles had become swollen, disrupted, and frequently were seen as amorphous formations. The chromatin substance of the nucleus had been transformed into a highly electron-dense substance of amorphous structure or formed lumps (Figs 4, 5 and 6). In lymphoid tissues still containing lymphocytes, the reticulocytes and erythrocytes seemed intact. However, when practically all the lymphocytes had disappeared, some reticulocytes or groups of reticulocytes showed signs of degeneration or necrosis. Based upon the severity of the observed changes, four grades were formed (Table II). Grade III and IV changes were only seen in mice that had died following treatment with large doses of either of the mycotoxins, whereas lesions of grade I and II were characteristic of mice treated with smaller doses; these animals survived until killed by bleeding. There was no difference between the examined organs (thymus, spleen, prepatellar and mesenteric lymph nodes) of the same animal in the severity of lesions.

Discussion

The two trichothecene mycotoxins (satratoxin H and T-2 toxin) produced similar changes (lymphocyte depletion and necrosis) in the lymphoid organs of mice dying of toxin treatment and those killed by bleeding after such treatment. The severity of the lesions depended on the toxin dose applied. Based upon the histopathological findings four grades (Grades I to IV) could be distinguished. Friend et al. (1983) also differentiated four severity grades of the regressive lesions induced in the thymus of mice by T-2 toxin. In our experiment, in the lymphoid organs of mice treated with the smaller doses depletion and necrosis affected only the lymphocytes. The reticulocyte framework of these organs remained intact, rendering possible their repopulation by lymphocytes. The fact that both the B-cell-dependent and T-cell-dependent regions of the lymphoid organs were affected indicates that in the toxin-treated animals both the humoral and the cell-mediated immune system were suppressed. Other investigators reported impaired resistance to various pathogens (including both extra- and intracellularly proliferating agents) in different

species of animals exposed to satratoxin H (Dankó, 1972; Dankó and Krasznai, 1976) and T-2 toxin (Friend et al., 1983; Corrier and Ziprin, 1986).

Based upon the LD_{50} values satratoxin H was five times as toxic as T-2 toxin.

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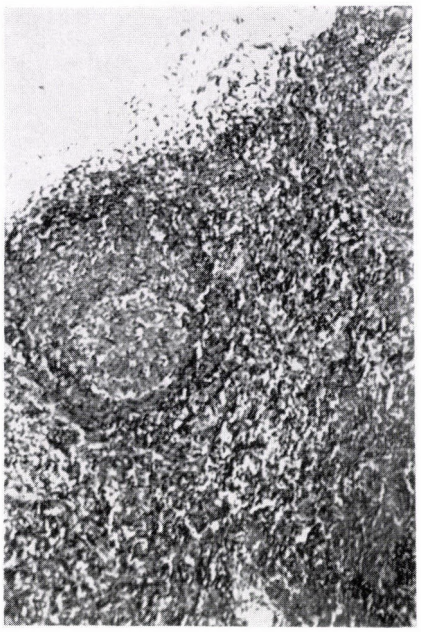


Fig. 1. Detail of lymph node from an untreated control mouse. Germinal centre and paracortical zone rich in lymphocytes. Haematoxylin and eosin (HE), ×160

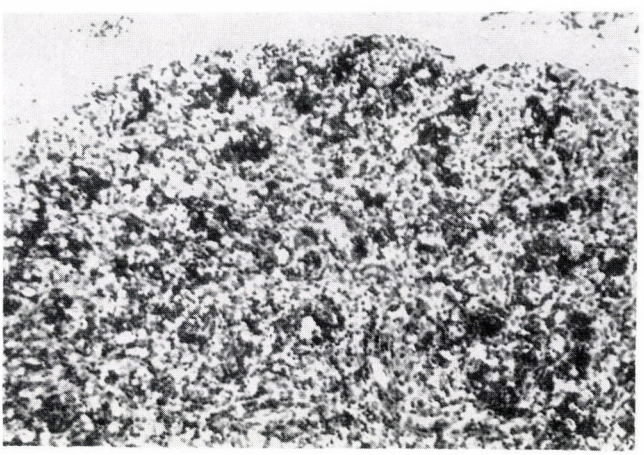


Fig. 2. Detail of lymph node from a mouse injected intraperitoneally with 1.60 mg/kg body mass satratoxin H. The animal died 24 h after treatment. Note lymphocyte depletion and indistinct structure in the germinal centres of the cortex and in the paracortical zones. HE, ×160

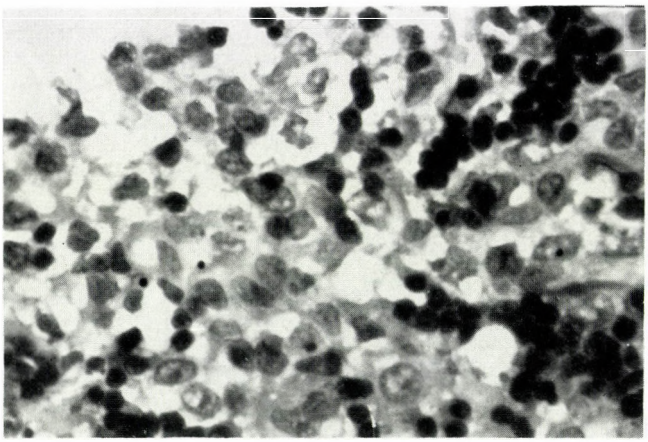


Fig. 3. Detail of Fig. 2. Note groups of degenerated lymphocytes in the reticulocytic framework of the lymph node. HE, ×400

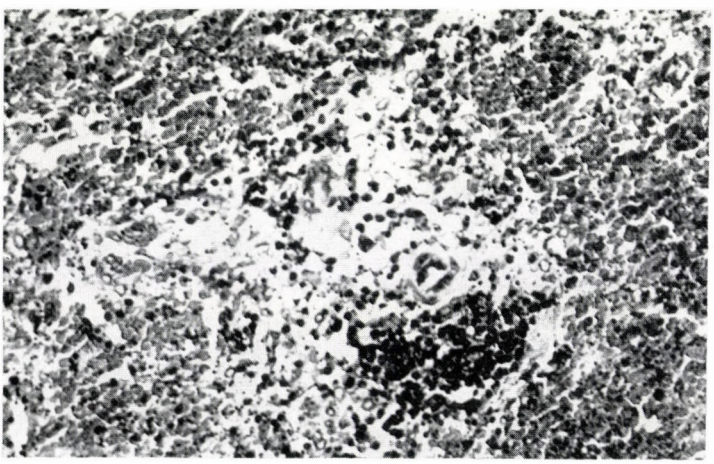


Fig. 4. Detail of the spleen from a mouse injected intraperitoneally with 4.0 mg/kg body mass T-2 toxin. The animal died 24 h after treatment. There are only few lymphocytes, with disintegrated nuclei, in the germinal centre and periarteriolar lymphocyte zone of the Malpighian body. HE, ×160

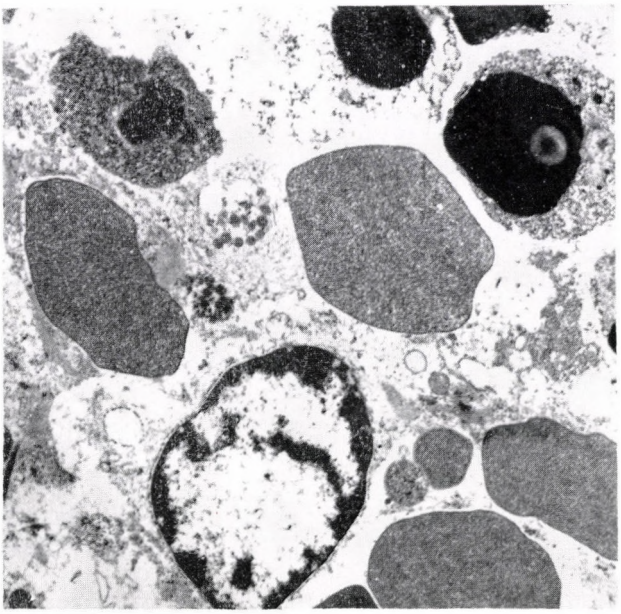


Fig. 5. Detail of the spleen from a mouse injected intraperitoneally with 1.75 mg/kg body mass satratoxin H and killed by bleeding 48 h thereafter. Top: necrotic lymphocytes with shrunken nuclei; middle and bottom: intact erythrocytes and reticulocyte. Electron micrograph, ×9600

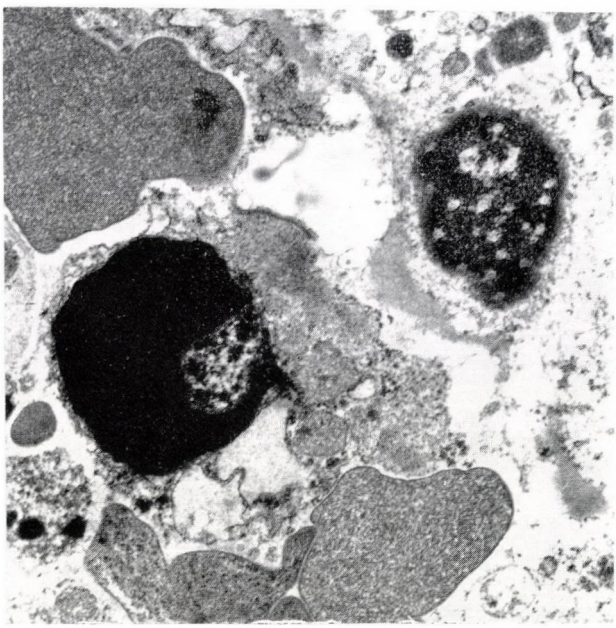


Fig. 6. Detail of the mesenteric lymph node from a mouse injected intraperitoneally with 4 mg/kg body mass T-2 toxin and killed by bleeding 48 h thereafter. The cell membranes of the two necrotic lymphocytes have undergone lysis, the structure of their cytoplasm has disintegrated, and the chromatin substance of their nuclei has become a strongly electron-dense mass devoid of distinct structure. Electron micrograph, ×14,300

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MILK PRODUCTION AND CERTAIN PARAMETERS OF ENERGY METABOLISM IN DAIRY COWS FED RATIONS OF VARYING ENERGY AND CRUDE PROTEIN CONTENTS AND FAT

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Twenty cows in mid-lactation were used in self-controlled experiments. After feeding the initial ration corresponding to the calculated energy and crude protein requirements of the dairy cows (100% E–100% P), four groups were formed as follows: Group A (80% E–100% P), Group B (100% E–120% P), Group C (80%E–120% P), and Group D (100% E–120% P). In Group D, 20% of the energy was contributed by fat. After a transition period the above rations were fed for two weeks.

Alterations in numerous blood parameters as well as in milk composition were indicative of a disturbed energy metabolism. The relative or absolute excess of crude protein resulted in increased blood and milk urea concentrations. A close negative correlation ($r = -0.90$) was found between milk urea concentration and the so-called urea fermentation potential (UFP) of the ration fed.

Energy deficiency was indicated by a decrease in milk production, blood glucose and milk protein concentration, and by increased total lipid and cholesterol levels of the blood plasma. The differences showed that the adverse effects of reduced energy and increased crude protein intake were added together. In Group C a pronounced increase of insulin concentration was observed, for which several possible explanations are suggested.

Effects of feeding fat (Group D) included a marked increase of total lipid and cholesterol concentrations of the blood plasma, a pronounced rise of milk urea and milk fat levels, and a decrease in milk protein and blood glucose concentrations. As regards fatty acid composition of the milk, the proportions of stearic acid and linoleic acid rose and that of oleic acid decreased.

Keywords: Dietary energy, crude protein, fat, metabolism, milk production, dairy cow.

In high-yielding dairy cows lactation performance and chances of a new conception greatly depend on the well-balanced nutrient supply (Farries, 1983; Gattinger and Baumgartner, 1985). As milk production rises, it becomes more and more difficult to provide the cows with the energy required (Lüpping and Kaufmann, 1979). Under conditions of the unique nitrogen metabolism characteristic of ruminants, energy-deficient feeding can cause protein deficiency even if the crude protein supply is adequate (Kaufmann, 1976; Miller et al., 1982). The amount of ammonia produced during rumen fermentation

depends on both the energy and crude protein content of the ration (Roffler and Satter, 1974), since these factors affect the protein supply status of ruminants in a complex manner.

Inadequate energy supply may lead to disturbances of the intermediary metabolism. In severe cases it may play a role in the development of numerous diseases such as diseases of the rumen, abomasum, liver and feet (Lüpping and Kaufmann, 1979; Stöber and Dirksen, 1982; Johnson, 1984). Changes in milk composition may indicate an unbalanced energy status even before the clinical symptoms of diseases appear. According to Farries (1983), the protein and fat contents or fatty acid composition of the milk undergo changes before a considerable decrease in milk yield occurs. The energy and protein supply status of dairy cows is well reflected in changes of milk urea concentrations (Eckart, 1980; Oltner and Wiktorsson, 1983).

Insulin, an anabolic hormone, exerts a pronounced influence on the energy balance of cows (Bines and Hart, 1982). It stimulates protein and lipid synthesis and inhibits the catabolism of these substances (Bassett, 1975). The concentration of insulin correlates with milk production (Hart et al., 1978). In ruminants not only volatile fatty acids (VFAs) but also ammonia plays a role in the stimulation of insulin release (Visek, 1984). The quantity of dietary energy and crude protein is of decisive importance in causing alterations in the blood concentrations of these compounds. The increased energy demands of high-yielding cows can less and less be satisfied by the conventional feeding methods. Increasing the proportion of concentrate in the ration is unfavourable from both the health and the economic aspect (Van Beukelen et al., 1982). A possible way of raising the energy content of the ration is to supplement it with fat (Palmquist and Jenkins, 1980).

Previous studies revealed that feeding fat exerted an adverse effect on ruminal fermentation processes (Henderson, 1973). Therefore, feeding fats in a quantity exceeding 5% of the dry matter of the feed is considered undesirable in recommendations made for ruminants (Kirchgessner, 1982; NRC, 1978). On the other hand, some researchers have observed that the energy supply status can be improved and milk production as well as milk fat content can be increased by feeding high fat rations to cows (Palmquist and Conrad, 1978; Smith et al., 1978; Kronfeld et al., 1980). Especially the so-called protected fats, which have been subjected to special treatments, seem to be favourable in ruminant feeding (Mattos and Palmquist, 1974; Blum et al., 1985), but untreated (natural) fats may also be beneficial when added to the ration in a proportion higher than the usual 2% of the dry matter (Storry et al., 1974; Heinrichs et al., 1981).

The objective of the present experiments was to study the effect of rations of energy and crude protein contents different from the requirements on some blood parameters reflecting the cows' energy and lipid metabolism

and on alterations in milk composition. Furthermore, the role of the energy source was studied, by ensuring part of the ration's energy content from natural (unprotected) fats.

Materials and methods

Twenty Holstein-Friesian R_1 cows of a large-scale dairy farm were used. The experiment lasted six weeks. The cows were between day 150 and day 240 of their lactation. Before the experiment average milk production was between 16.3 and 24.0 FCM kg/day. The cows were allotted to four experimental groups of five animals each (Groups A, B, C and D). Average milk production as well as the number and phase of lactation were the same in the four groups.

The experimental period consisted of three phases in which different feeding regimes were used (Table I). In the first phase (days 1 to 14) all experimental cows received a so-called "basal ration" corresponding to their body mass and milk production level (NRC, 1978). Twenty per cent of the energy requirement was met by mixing corn-starch in the concentrate. In the second ("transition") phase (days 15 to 28) different feeding regimes were introduced in the four groups. From day 15, starch was omitted from the ration fed to cows of Group A; thus, the energy content of their ration decreased to 80% of the requirement. Crude protein intake was left unchanged. Cows of Group B received urea in amounts increasing from day to day: as a result, from day 28 of the experiment these cows were given 20% excess crude protein. Similarly to Group B, in Group C the N content of the ration was increased by 20%, while its energy content was reduced to 80% by omitting starch. The crude protein content of the ration fed to Group D cows was gradually raised to 120% of the calculated requirements, and 20% of the energy was contributed by Energomix (a pulverized fat preparation containing 50% stabilized and emulsified animal fat and 50% flaked corn). In the third phase of the experiment (days 29 to 42) the rations described above were fed throughout. When needed, the quantity of the rations was corrected for the cows' milk production.

Blood samples were taken at the end of the first phase (sampling time 1), in the middle of the second phase (sampling time 2), and at the end of the third phase (sampling time 3), always before the morning feeding. The samples were assayed for blood glucose (G) concentration, total lipid (TL) and total cholesterol (TCh) concentration of the blood plasma, and for AST activity. Plasma urea (PU) concentration was determined by a specific enzymatic method (Fawcett and Scott, 1960) based on Berthelot's reaction. The insulin concentration of the blood plasma was determined with a hormone analysis kit based on ^{125}I-labelled insulin (Chattora and Watts, 1986). On two occasions

(at sampling times 1 and 3) the fatty acid composition of blood plasma lipids was examined by gas chromatography (Husvéth et al., 1982).

Milk production was recorded, and milk fat and milk protein contents were determined. Milk urea (MU) concentration was also determined by Berthelot's method, using milk whey. To obtain milk whey, milk samples were deproteinized with uranyl acetate and centrifuged at 8000 rpm for 10 min.

Also on two occasions (sampling times 1 and 3), we determined the quantity of long-chain fatty acids in the milk samples. For this purpose, milk lipids were extracted (Husvéth et al., 1982) and, with HCl-methanol, fatty acid methyl esters were produced as described by Christie (1973). The test material was dissolved in chloroform and the fatty acids were separated with a Packard-Becker 419 type gas chromatograph. CHROMOSORB WHP of 100 to 120 mesh was used as solid support and 10% CS-10 as liquid phase. Statistical evaluation of the data obtained within and among the experimental groups at the different testing times was performed by Duncan's test, analysis of variance, and correlation and regression calculations (Steel and Torrie, 1960; Sváb, 1981).

Results

Alterations in G, TL, TCh and PU concentrations and AST activity in the various experimental groups are shown in Table II.

Blood G concentration decreased by 20 to 30% in all four experimental groups. At sampling time 2 the values obtained for Group C were significantly different from those of Group A (2.32 and 2.66 mmol/l). Blood G concentrations lower than the baseline level persisted up to the end of the experiment; however, they did not drop below the lower limit of the physiological range.

At sampling time 2, *TL concentration* was increased in Groups A, B and D, and practically unaltered in Group C (2.21–2.28 g/l). There were significant differences between several of the groups. The greatest increase in TL was found in Group D (2.71 to 3.99 g/l). At sampling time 3, in Groups A and C rather low TL concentrations were measured; however, even these exceeded the baseline level by more than 50%. In Group D an even higher TL concentration was found at sampling time 3 (4.14 g/l), as compared to time 2.

TCh concentration increased in all groups during the experiment. However, in the two groups fed an energy-deficient diet (Groups A and C) this increase was much less expressed (3.18 and 3.04 mmol/l, respectively). In Group D TCh increased to nearly two and a half times that of the baseline level (from 2.28 mmol/l to 5.28 mmol/l).

Blood plasma AST activity remained within the physiological range throughout the experiment in all experimental groups (between 19.0 and 31.4 U/l).

Baseline *PU concentration* (at the first sampling) was around 4 mmol/l. By the second sampling in Group A it had increased by more than 50% (from 4.29 to 6.82 mmol/l). A similar, almost twofold, increase in PU concentration was found in Groups B and C (from 4.15 to 8.10 mmol/l and from 4.23 to 8.28 mmol/l, respectively). The most pronounced concentration change (about 120%) was found in Group D, in cows that had received fat supplementation (from 3.96 to 8.52 mmol/l).

Changes in *plasma insulin concentration* are shown in per cent of the baseline values (Fig. 1). In Group A insulin concentration decreased to 29% of the initial value. In Group B a slight, transient decrease (sampling time 2) was followed by a 86% increase up to the end of the experiment. The most striking change was found in Group C, where at the end of the experiment insulin concentration was 4.4 times higher than the original value. In Group D the concentration of this hormone remained practically unaltered.

Among the blood plasma lipids, of the long-chain fatty acids palmitic acid ($C_{16:0}$), stearic acid ($C_{18:0}$), oleic acid ($C_{18:1}$) and linoleic acid ($C_{18:2}$) occurred in the highest proportion (Table III). The proportion of myristic acid and palmitic acid decreased significantly in all groups and a slight reduction in the proportion of stearic acid was found in all groups but Group A.

Table I

Composition and nutritive value of the rations

Feeds and their parameters	Groups and rations				
	Basal ration	A	B	C	D
Energy : protein ratio, %	100 : 100	80 : 100	100 : 120	80 : 120	100 : 120*
Corn silage, kg	18.0	18.0	18.0	18.0	18.0
Alfalfa hay, kg	4.5	4.5	4.5	4.5	4.5
Concentrate, kg	2.0	2.0	2.0	2.0	2.0
Mineral supplement (Boscaphos II), g	140	140	140	140	140
Starch, kg	1.5	—	1.5	—	—
Urea, g	—	—	120	120	120
Energomix-50, kg	—	—	—	—	0.9
Dry matter, kg	11.93	10.58	12.05	10.70	11.39
Crude fibre, kg	2.44	2.44	2.44	2.44	2.44
NE_l, MJ**	84.70	67.76	84.70	67.76	84.70
Crude protein, g***	1743	1743	2092	2092	2092
Crude fat, g	245	245	245	245	650
Ca, g	99	99	99	99	99
P, g	55	55	55	55	56
UFP, g****	−91	−115	−211	−235	−230

* 20% of the energy given by fat; ** milk-producing net energy; *** N×6.25; **** urea fermentation potential (Burroughs et al., 1974)

Table II

Blood parameters tested at the first, second and third sampling time (mean ± SD)

Group	Blood glucose (G) (mmol/l)			Total lipid (TL) (g/l)			Total cholesterol (TCh) (mmol/l)			Urea (PU) (mmol/l)			AST activity (U/l)		
	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
A	3.12 ±0.16	2.66^{c} ±0.22	2.66^{c} ±0.24	2.21 ±0.30	3.07^{b} ±0.27	3.22^{b} ±0.39	1.5 ±0.27	3.0^{c} ±0.56	3.18^{c} ±0.57	4.29 ±0.50	6.82^{c} ±0.7	6.80^{c} ±0.4	29.8 ±7.1	23.2 ±5.9	18.4 ±1.5
B	3.06^{c} ±0.25	2.40^{b} ±0.31	2.54^{b} ±0.15	2.50 ±0.43	3.24^{ab} ±0.39	3.56^{ab} ±0.32	2.04 ±0.61	3.86^{c} ±0.34	3.70^{c} ±0.57	4.15 ±0.54	7.95^{c} ±1.3	8.10^{c} ±0.67	25.0 ±4.0	31.1 ±3.6	19.2 ±3.5
C	3.00 ±0.21	2.32^{b} ±0.16	2.70^{b} ±0.19	2.21^{ab} ±0.39	2.28^{ab} ±0.47	3.34 ±0.36	1.6 ±0.22	2.88^{a} ±0.68	3.04^{a} ±0.55	4.23 ±0.56	9.30^{ac} ±0.53	8.28^{ac} ±0.66	29.0 ±4.9	25.8 ±4.4	20.4 ±1.6
D	3.24 ±0.44	2.60^{b} ±0.36	2.72^{b} ±0.20	2.71 ±0.32	3.99^{c} ±0.31	4.14^{c} ±0.24	2.28 ±0.68	4.3^{c} ±0.67	5.28^{c} ±0.48	3.96 ±0.55	7.78^{c} ±0.96	8.52^{c} ±0.27	30.6 ±4.6	27.4 ±5.4	19.0 ±4.4
Significance level of the inter-group differences	NS	A–C	NS	NS	A–C A–D C–D B–C B–D	A–D B–D C–D	NS	A–D B–C C–D	A–D B–C B–D C–D	NS	A–C C–D	A–B A–C A–D	NS	NS	NS

Comparison of the values obtained for the same parameter at different sampling times: values indicated with the same superscript *do not* differ significantly; ^{a}: $P < 0.05$; ^{b}: $P < 0.01$; ^{c}: $P < 0.001$

Table III

Fatty acid composition of the blood plasma (mean ± SD)

Fatty acid	Experimental groups and sampling times								Comparison of the groups and of two sampling times by *t* test
	A		B		C		D		
	1	3	1	3	1	3	1	3	
$C_{12:0}$	4.09 ± 1.65	13.0 ±3.19	4.9 ±0.14	9.76 ± 4.04	2.46 ±1.93	9.27 ±0.51	4.23 ±0.97	7.83 ±2.06	
$C_{14:0}$	12.32[a] ± 3.61	5.11[a] ±0.80	10.51[b] ±1.79	4.59[b] ± 0.78	9.31[c] ±3.52	4.91[c] ±1.04	9.92[d] ±2.04	3.79[d] ±1.11	[a]: $P < 0.01$; [b]: $P < 0.001$ [c]: $P < 0.001$; [d]: $P < 0.001$
$C_{16:0}$	10.74[a] ± 1.90	7.20[a] ±0.26	10.50[b] ±1.69	8.23[b] ± 1.24	13.75[c] ±5.82	8.00[c] ±0.15	12.82[d] ±2.90	8.25[d] ±1.12	[a]: $P < 0.01$; [b]: $P < 0.05$ [c]: $P < 0.05$; [d]: $P < 0.05$
$C_{16:1}$	3.15 ± 0.53	2.36 ±0.34	3.19 ±0.88	2.97 ± 0.50	3.56 ±0.28	2.81 ±0.43	4.43 ±0.89	3.23 ±0.17	
$C_{18:0}$	10.76 ± 2.68	12.02 ±0.78	12.96 ±1.03	11.87 ± 0.49	13.26 ±1.38	12.54 ±0.78	13.42 ±1.69	11.87 ±0.51	
$C_{18:1}$	8.3 ± 2.58	8.95** ±0.73	9.09 ±1.37	9.42 ± 1.51	11.70 ±2.09	10.28 ±0.50	9.50 ±1.31	10.81** ±1.08	** A—D $P < 0.05$
$C_{18:2}$	30.48 +11.75	31.98* +2.25	31.29 +3.10	31.15* +11.74	26.86 +6.45	31.67* +0.87	22.33 +8.31[a]	35.06* +2.61[a]	* A, B, C—D $P < 0.05$ [a]: $P < 0.05$
$C_{18:3}$	4.75 ± 0.36	4.12 ±0.41	5.39 ±1.16	4.48 ± 0.69	4.09 ±1.41	4.69 ±0.47	5.62 ±1.66	4.91 ±0.79	
$C_{20:3}$	3.23 ± 1.32	2.61 ±0.58	2.78 ±0.39	2.96 ± 0.65	3.22 ±0.54	2.81 ±0.40	4.15 ±1.24	2.88 ±0.88	
$C_{20:4}$	4.65 ± 2.07	3.88 ±0.86	3.63 ±0.47	4.73 ± 1.40	4.21 ±1.33	4.48 ±0.35	4.90 ±0.14	3.32 ±0.12	
$C_{24:0}$	3.56 ± 2.4	4.58 ±1.07	2.73 ±1.74	6.12 ± 1.63	3.73 ±0.2	4.55 ±1.46	4.01 ±0.77	4.38 ±0.27	

Table IV

Changes in milk production, milk protein concentration and butterfat percentage (mean ± SD)

	Parameters and sampling times									Significance levels of the difference between means of the different samplings
	Milk production, kg/day			Milk protein, %			Butterfat, %			
	1	2	3	1	2	3	1	2	3	
A	17.00^a $\pm 2.03^b$	18.36^a ± 2.42	13.20^b ± 2.20	4.17 ± 0.23	4.37 ± 0.75	4.27 ± 0.45	3.49^c ± 0.25	3.68 ± 0.50	3.12^c ± 0.80	$^{a, b, c, d}$: $P < 0.05$ ^{c}: $P < 0.001$
B	15.22^a ± 1.10	12.16^a ± 2.58	12.00^b ± 2.65	3.95^c $\pm 0.56^d$	4.33^c ± 0.64	4.54^d ± 0.34	3.37 ± 0.17	4.24 ± 0.36	3.63 ± 0.67	$^{a, b, c}$: $P < 0.05$ ^{d}: $P < 0.001$
C	15.30^a $\pm 0.83^b$	11.24^c ± 2.4	10.20^b ± 2.95	4.62^c ± 0.41	4.17 ± 0.40	4.53 ± 0.49	3.60 ± 0.32	3.78 ± 0.63	3.63 ± 0.29	$^{a, b, c}$: $P < 0.01$
D	17.26^a $\pm 2.03^b$	14.32^a ± 1.21	14.00^b ± 1.00	4.64^c $\pm 0.39^d$	3.86^c ± 0.27	3.74^d ± 0.21	3.29^e $\pm 0.37^f$	3.88^e ± 0.52	3.81^f ± 0.56	$^{a, b, c, d, e, f}$: $P < 0.05$
Significance level of the inter-group differences	NS	C—D $P < 0.05$	C—D $P < 0.05$	NS	NS	A—D $P < 0.05$ B—D $P < 0.01$ C—D $P < 0.05$	NS	A—B $P < 0.01$	NS	

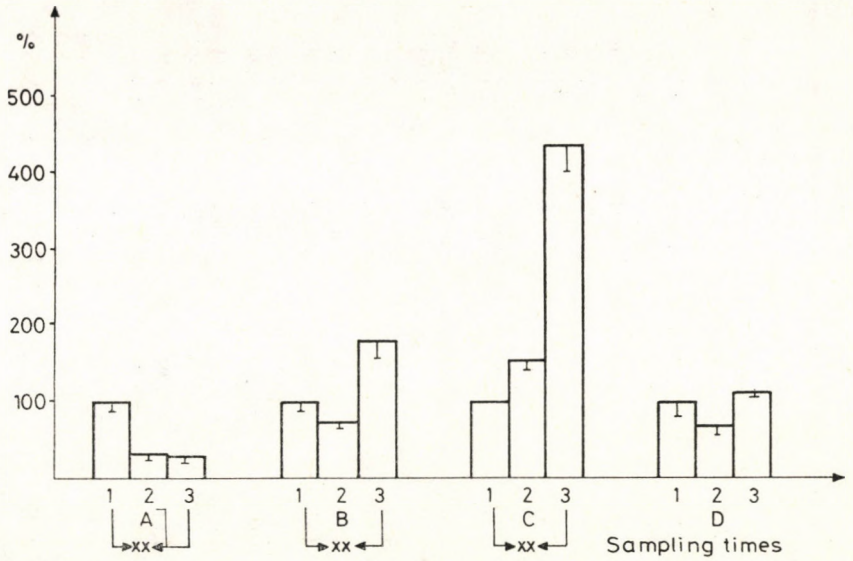


Fig. 1. Blood plasma insulin concentrations at the three sampling times, expressed as per cent of the baseline value (** significance level of the difference between Groups A, B and C: $P < 0.01$)

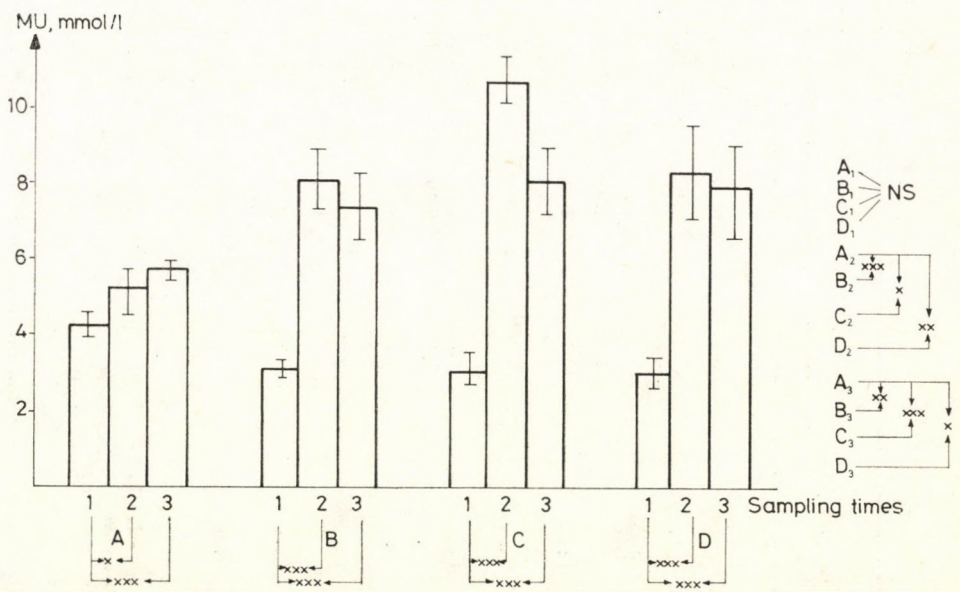


Fig. 2. Milk urea (MU) concentrations at the three sampling times (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$) in Groups A, B, C and D

Table V

Fatty acid composition of the milk (mean ± SD)

Fatty acid	Groups and sampling times							
	A		B		C		D	
	1	3	1	3	1	3	1	3
$C_{10:0}$	3.23 ±0.37	3.51 ±0.28	2.62 ±0.80	3.37 ±0.86	2.79 ±0.45	2.93 ±0.51	3.31 ±0.59	3.55 ±0.94
$C_{12:0}$	4.09 ±0.69	5.17 ±0.43	3.67 ±0.56	4.96 ±0.99	3.36 ±0.48	4.62 ±0.85	3.94 ±0.59	5.14 ±0.41
$C_{14:0}$	12.36 ±1.49	14.64 ±1.03	11.58 ±0.68	12.19 ±1.18	12.02 ±0.68	14.59 ±3.24	12.69 ±1.09	13.08 ±1.07
$C_{15:0}$	3.13 ±0.59	4.56 ±0.86	3.29 ±0.46	5.10 ±1.14	3.49 ±0.54	4.45 ±0.81	3.08 ±0.73	5.38 ±1.18
$C_{16:0}$	33.81^{B} ±2.61	30.42^{abc} ±1.14	34.37^{A} ±5.42	27.71^{Aa} ±2.22	35.46^{A} ±1.50	27.00^{b} ±1.42	30.70^{A} ±2.97	26.68^{C} ±1.64
$C_{16:1}$	3.46 ±0.57	4.02 ±0.46	3.46 ±0.29	4.97 ±0.59	3.47 ±0.75	4.76 ±1.01	2.87 ±0.40	4.37 ±0.43
$C_{18:0}$	10.12 ±2.42	11.14 ±1.87	9.50 ±1.34	11.57 ±1.74	9.07 ±0.51	10.83 ±0.99	9.16 ±0.72	12.53 ±1.38
$C_{18:1}$	22.79 $\pm 3.14^{C}$	18.98 $\pm 1.24^{C}$	24.77^{B} ±4.48	19.91^{B} ±2.59	23.78^{C} ±1.65	19.27^{C} ±1.29	22.30^{C} ±2.04	19.08^{C} ±0.90
$C_{18:2}$	1.90 $\pm 0.59^{D}$	2.52^{d} $\pm 0.32^{D}$	2.52 ±1.03	3.20 ±0.78	2.40^{D} ±0.74	3.42^{d} $\pm 0.38^{D}$	1.87^{D} ±0.49	3.41 $\pm 0.36^{D}$
$C_{18:3}$	1.48 ±0.40	1.46 ±0.30	1.06 ±0.66	2.00 ±0.66	1.61 ±0.31	1.97 ±0.77	7.0 ±0.15	1.78 ±0.32
Significance level of the difference between samplings	^{A}: $P < 0.05$ ^{B}: $P < 0.01$ ^{C}: $P < 0.01$ ^{D}: $P < 0.01$		^{A}: $P < 0.05$ ^{B}: $P < 0.05$		^{A}: $P < 0.001$ ^{B}: $P < 0.01$ ^{C}: $P < 0.01$ ^{D}: $P < 0.05$		^{A}: $P < 0.001$ ^{B}: $P < 0.01$ ^{C}: $P < 0.01$ ^{D}: $P < 0.001$	

Significance level of the inter-group differences: ^{a}: $P < 0.05$; $^{b, c, d, e}$: $P < 0.01$

In Group D the proportion of oleic acid increased. A considerable rise in the proportion of linoleic acid occurred in Groups C and D (from 26.8 to 31.6% and from 22.3 to 35.1%, respectively).

The *production parameters* studied are shown in Table IV. A milk production drop was found in all four groups at sampling time 2, the degree of which decrease, however, varied by group. In Group C the milk yield kept decreasing further up to the end of the experiment.

At the beginning of the experiment, *milk protein concentration* was between 3.95 and 4.65%. In Group A it did not change considerably even later on. On the other hand, in Group B a 20% rise (from 3.95 to 4.64%) was observed. In Group C, after a transient decrease (from 4.62 to 4.17%), milk protein concentration returned to the initial level (4.58%). In Group D there was a big difference between milk protein values measured at the first and third sampling: a 20% drop occurred from 4.64 to 3.74%, which is significantly lower than the average values obtained for the other groups.

Butterfat content was between 3.29 and 3.57%. In Group A it decreased from 3.68 to 3.12%, while in Groups B and C nearly identical values were measured at the beginning and at the end of the experiment (3.57, 3.60 and 3.63%). On the other hand, in the milk of Group D cows butterfat content underwent a nearly 20% rise (from 3.29 to 3.84%).

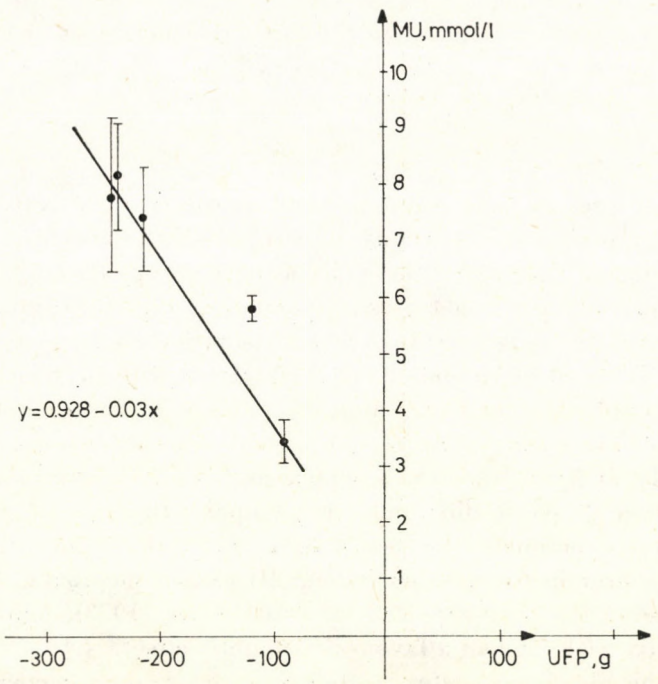


Fig. 3. Relationship between urea fermentation potential (UFP) of the feeds and milk urea (MU) concentrations

Milk urea (MU) concentration was in close correlation ($r = +0.86$; $P < 0.01$) with the PU level. At the beginning of the experiment the group averages ranged between 3.04 and 4.24 mmol/l (Fig. 2). In Group A, MU concentration increased by nearly 40%; in the other groups a still greater concentration change was found: in Groups B and C, MU increased by about 150 and by more than 200%, respectively, by the time of the second sampling. In Group D a similar concentration change was observed as in Group B.

In the *fatty acid composition of milk lipids* (Table V) considerable alterations occurred in the proportion of palmitic acid and oleic acid. The proportion of both of these fatty acids decreased in all four groups; in Groups B and C the change was more expressed than in Groups A and D. In Group D only the contribution of stearic acid and linoleic acid increased considerably. Comparing the various groups at the end of the experiment, only in Group A did the proportion of palmitic acid exceed 30%, whereas in the same group the proportion of linoleic acid remained significantly ($P < 0.01$) lower.

We examined the connection between milk urea concentration (y, mmol/l) of cows in the different groups and the UFP values (x, g) of the five different feeds formulated by us (basal, A, B, C and D). UFP (urea fermentation potential) expresses the energy and protein ratios of the different diets and mixed feeds, and the digestibility of their protein in the rumen (Burroughs et al., 1974). A close negative correlation ($r = -0.90$; $P < 0.001$) was found between these two parameters (Fig. 3). In the range studied by us, the linear relationship could be described by the equation $y = 0.928 - 0.03x$.

Discussion

In the present experiment we studied the effect of rations with energy and crude protein concentrations different from the optimum on dairy cows in mid-lactation. In one of the experimental groups (Group D) most of the energy requirement was met by a supplement of high fat content added to the ration. Our choice fell on this period of lactation to avoid the possible confounding effects of hormonal changes associated with parturition and involution. For crude protein overfeeding the excess N was provided by adding, after an adequate adaptation period, urea to the concentrate. Urea is rapidly and completely hydrolysed in the rumen and is suitable for revealing relationships between N overfeeding and energy supply status.

In our experiments neither a 20% energy deficiency (Group A) nor a 20% crude protein overfeeding (Group B) caused metabolic disorders. Similarly to the observation reported by Smith et al. (1978), supplementation of the feed mix with fat had a favourable dietetic effect.

Of the blood parameters tested, G concentration remained within the physiological range throughout. Neither did the varying energy supply level

result in significant differences between the groups. Our results support the opinion of Reid and Hinks (1963), who claimed that in ruminants blood glucose level was not suitable for assessing the energy supply status.

The transient decrease in blood G level observed in Groups B and C may have been induced by supplementing the feed with urea, as it had been found earlier in sheep (Leonard et al., 1977).

The striking rise of the TL level found in Group D can be attributed to fat supplementation (Astrup et al., 1976; Palmquist and Conrad, 1978). The concentration rise observed in Group A is a concomitant of energy deficiency. The increase of the TL level in Group B is remarkable since it was accompanied by a simultaneous rise in the concentration of insulin, a hormone of antilipolytic property. Presumably, the ammonia derived from the dietary urea must have resulted in an enhanced adrenaline production. As opposed to insulin, adrenaline has a lipolytic effect (Symonds et al., 1981). A similar situation occurred in Group C, where energy deficiency was coupled with crude protein overfeeding. The antilipolytic effect of the high insulin concentrations measured in that group must have been compensated, by the time of the third sampling, by hormones stimulating lipid mobilization, or the large amounts of ammonia produced may have blocked the action of insulin (Visek, 1984).

As opposed to others (Tainturier et al., 1984), we observed a rise in the TCh level in all four groups. Namely, cholesterol concentration was usually the highest at the end of the first third of lactation (Kweon et al., 1986), and the lowest after parturition (Pethes et al., 1984). In our study, already at sampling time 1 we found lower TCh concentration than that measured in the same phase of lactation by others (Kweon et al., 1986). Supplementation of the feed with fat raises the cholesterol concentration of the blood plasma in ruminants (Sharma et al., 1978; Wrenn et al., 1978), as it was seen in Group D of our experiment. In contrast with what is seen in monogastric animals, in ruminants the fat added to the diet is not ketogenic. In animals with a composite stomach, decomposition of chylomicrons and VLD lipoproteins takes place in extrahepatic tissues, in dairy cows principally in the udder (Palmquist and Mattos, 1978).

AST activity, which is an indicator of possible hepatic dysfunctions, did not change considerably in either of the groups during the experiment. This allows us to conclude that the experimental diets did not cause appreciable damage to the liver parenchyma. Thus, the status of the liver did not influence milk and plasma urea (MU and PU) level. Alterations in MU can, therefore, be considered a good indicator of the energy and crude protein supply status. The MU values measured at the beginning of the experiment (3.04–4.24 mmol/l) fall in a range indicative of a well-balanced energy and N supply meeting the requirements (Eckart, 1980; Oltner and Sjannja, 1982). In Group A the rise of urea concentrations was less expressed than in the groups which were overfed with crude protein. The greatest concentration rise was produced by

rations deficient in energy but containing supraoptimal quantities of crude protein (simultaneous relative and absolute N overfeeding). Cows given fat supplementation had high milk and plasma urea levels, suggesting that although fat supplementation of the diet can improve the energy supply status of ruminants (Kronfeld, 1976; Baldwin et al., 1985), it has no influence on the protein synthesizing activity of the rumen microflora (Blum et al., 1985; Lüpping and Kaufmann, 1979; Rohr et al., 1978). The close negative correlation found between the UFP of the feeds and MU concentration proves that, within the range studied by us (UFP = 90–235; MU concentration = 2.5–10 mmol/l), MU concentration is a good indicator of the energy and protein supply status of the rumen microflora.

In Group D, fat supplementation of the diet exerted a significant effect on the fatty acid composition of the blood plasma. After the ingestion of large amounts of fat, the proportion of palmitic acid was found to decrease in calves (Wrenn et al., 1978). Theoretically the uptake of excess fat might also have accounted for the increased proportion of linoleic acid, though most of the unsaturated fatty acids getting into the rumen with the feed become saturated by microorganisms (Kepler et al., 1966), a process resulting in an increased proportion of stearic acid. When large amounts of concentrate are fed, the saturating activity of microorganisms decreases (Palmquist and Jenkins, 1980) and, thus, part of the fat leaves the forestomachs in unsaturated state. In the beginning of the present experiment rather large quantities of starch were fed (corresponding to 20% of the dietary energy). Supposedly, this could have reduced the saturating activity of bacteria.

The increased proportion of linoleic acid observed in Group C might indicate an enhanced lipid mobilization from the fat depots, a process observed by us in energy-deficient sheep earlier (Magdus et al., 1985). On the other hand, the characteristic, opposite concentration changes of stearic acid and oleic acid, which are typical of early lactation and energy deficiency, were not observed in any of the groups (Husvéth et al., 1982).

The progress of lactation played a role in the reduction of milk production in all four groups. This fact, however does not affect the interpretation of our data, since the milk production drop was accompanied by a slight decrease of milk urea concentration (by 0.06 mmol/l per one kg of milk; Kirchgessner et al., 1986).

The more pronounced milk production drop observed in Group C may have been attributable, at least in part, to the marked disproportionateness existing between the energy and crude protein content of the feed. In Group D fat supplementation somewhat increased the persistence of milk production (Maynard et al., 1941; Heinrichs et al., 1981).

In Group A fed an energy-deficient diet we could not observe the decrease in milk protein concentration reported by Kirchgessner et al. (1986). In Group

B abundant crude protein and satisfatory energy supply led to an increase in milk protein concentration (Mac Leod et al., 1984). In ruminants blood glucose and volatile fatty acids constitute the main energy source of milk protein synthesis (Pelissier et al., 1986). Fat feeding decreases the concentrations of the above two metabolites and does not promote microbial protein synthesis in the rumen (Palmquist and Moser, 1981). Presumably, this is why we found in Group D a decrease in milk protein concentration, which was observed by others when feeding soybean oil or tallow to cows (Clapperton and Steele, 1985). Despite the rather large drop of milk production, neither milk protein nor butterfat content changed in Group C.

The energy-deficient diet fed in Group A resulted in decreased butterfat content. On the other hand, in Group D the greater fat intake led to a considerably increased butterfat percentage (Palmquist and Conrad, 1978; Wrenn et al., 1978). This was indicated by the greater increase of the proportion of stearic acid and, presumably, linoleic acid than in the other groups (maize, which was the carrier of the fat supplement, is rich in linoleic acid). In Groups B and C the elevated insulin concentration accounted for the decreased proportion of palmitic acid and oleic acid, by inhibiting lipid mobilization from the fat depots.

Alterations of insulin concentration in different directions were caused by a combined action of several factors. In Group A, decreased energy intake and reduced formation of propionic acid (omission of starch from the basal diet) played a role (Bines and Hart, 1982). In Groups B and C the large amounts of ammonia produced in the rumen must have enhanced the secretion of insulin, induced insulin resistance and decreased the metabolic clearance of the hormone (Visek, 1984).

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DIGESTION AND FAECAL MINERAL CONTENT OF VIRGINIAMYCIN-FED RABBITS EXPOSED TO PHYSIOLOGICAL AND SIMULATED STRESS

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The effect of virginiamycin on nutrient digestibility and faecal mineral content was studied in rabbits. The dose was 30 mg/kg feed. In part of the experimental period, the rabbits received ACTH, 0.5 IU/kg body mass. Supplementation of the feed with virginiamycin improved protein digestibility by about 4%. At the same time, the digestibility of crude ash decreased by 10%. The digestibility coefficients of energy and organic matter did not change. ACTH treatment did not influence nutrient digestibility, either in rabbits fed the basal diet or in those given the virginiamycin-supplemented diet. Virginiamycin supplementation, as well as ACTH treatment, altered the mineral composition of the faeces (K^+, Na^+, Cu^{2+}) and, particularly, the ratio of certain mineral elements (Na : K).

Keywords: Virginiamycin, stress, digestibility, faeces mineral, rabbit.

When added to the feed, virginiamycin improves feed utilization in poultry and swine (Powley et al., 1981; Miles et al., 1984), increases the average daily body mass gain and reduces losses caused by enteric disorders (Bergamaschi, 1979). At a dose rate of 20 mg/kg of feed, virginiamycin was found to exert a beneficial effect on the production parameters in rabbits (Okerman, 1982). Contrarily, Solca et al. (1980*b*) reported that virginiamycin failed to alter the average daily body mass gain although it did improve feed efficiency. Carregal et al. (1984) failed to demonstrate any beneficial effect of virginiamycin on either body mass gain or feed efficiency. Vandaele (1980) reviewed the studies conducted on virginiamycin by various researchers and made the following statements: when mixed in the feed at a dose rate of 20 mg/kg, the antibiotic improved the average daily body mass gain and feed utilization of growing rabbits by 5 to 10%. Pregnant does fed on virginiamycin-supplemented diets bring forth young rabbits weighing 5 g more at birth. The compound is practically atoxic: no adverse consequences occur even after five- to ten-fold overdosing. Experiments with poultry, calves and rabbits have shown that no virginiamycin residues are left over in the meat.

Investigations into its mechanism of action have revealed that virginiamycin reduces the net mass of the small intestine and caecum (King, 1974) and the thickness of their mucous membrane (King, 1971; Costa-Battlori, 1976). According to the detailed analysis performed by Solca et al. (1980*a*),

primarily the wall of the intestinal portions playing an important role in absorption becomes thinner, indicating the healing of a subclinical inflammation.

Ruffini-Castrovilli et al. (1980) studied the effect of virginiamycin on the food-hygienic value of table rabbits. They found that, though virginiamycin changed the fatty acid composition of the fat depots, this change was not detrimental to the kitchen value of the rabbits.

In the digestibility studies performed by Rigoni et al. (1980) virginiamycin improved the digestibility of protein, fat and gross energy, and slightly worsened that of crude fibre. The N retention and fat incorporation of growing rabbits increased by 20% and 4%, respectively, a change favourable for the consumer. The studies done by Parigi-Bini (1981) on weanling rabbits also showed that virginiamycin improved energy and crude protein digestibility and increased N retention.

The studies mentioned above had been carried out under good, well-controlled experimental conditions. Under conditions of up-to-date animal production animals are frequently exposed to stressors and get into a state of stress (Marina and Tonkonglas, 1982). Extreme ambient temperatures (Christison and Johnson, 1972), malnutrition (Smith et al., 1975) or overcrowding (Maertens and DeGroote, 1984) may act as stressors. Elevated blood glucocorticoid level is known to be one of the characteristic features of stress condition (Selye, 1956). This impairs the organism's resistance against infection (Lu et al., 1982) and may lead to alterations in the composition of the intestinal flora (Kovács et al., 1980).

In the present work we examined the effect of virginiamycin on the rabbits' digestion not only under optimum conditions but also in stress condition elicited by administering ACTH, a method widely used for simulation of stress (Friend et al., 1977; Davies and Siopes, 1985). Considering that both the elevated blood corticosteroid level and virginiamycin feeding can alter the absorption of certain minerals (Roe, 1974; Henry et al., 1986), the mineral composition of faecal samples collected in various stages of the experiment was determined.

Materials and methods

Ten female rabbits of the Californian breed, aged 4–5 months and 2.9 $\pm$ 0.3 kg in body mass, were used. The animals were assigned to two main experimental groups: control (C) and antibiotic-treated (A). The diet of the latter rabbits contained 30 mg virginiamycin (Smith and Kline, Brussels) per kg. The granulated feed of 0.5 $\times$ 1.0–1.2 cm pellet size consisted of 11% corn, 15% wheat, 12% oats, 5% wheat bran, 14% extracted sunflower meal,

2% extracted soybean meal, 35% alfalfa meal, 2% dried sugar-beet pulp, 2% Favorit-50 (a fat preparation), 1% AP-17 (calcium phosphate), 0.5% sodium chloride and 0.5% vitamin-microelement premix (BCR). The chemical composition of the diet is shown in Table I.

The two groups of rabbits, each consisting of 5 animals, were placed into metabolic cages and offered feed and drinking water ad libitum. The

Table I

Chemical composition of the experimental diets

Constituents*	Control (C)	Antibiotic-treated (A)
Dry matter, %	89.20	89.30
Crude ash, %	4.85	4.90
Crude protein, %	20.48	20.53
Crude fibre, %	11.70	11.65
Crude fat, %	5.0	4.9
N-free extract, %	57.97	58.02
Gross energy, MJ/kg	18.61	18.62

* Dry matter is expressed in per cent of the diet, all other values are expressed in per cent of dry matter

animals were weighed individually every Monday and Friday. The experiment lasted six weeks. The first week was the preparatory phase, whereas the 2nd and 3rd weeks represented the main (collection) phase of the basic digestibility study. In the fourth week ACTH treatment of all rabbits was started, with daily intramuscular doses of 0.5 IU/kg body mass (Exactin injectable, G. Richter Pharmaceutical Works, Budapest). The fourth week was regarded as transition period. In the subsequent two weeks ACTH treatment was continued and a new digestibility study was performed to elucidate the relationship of experimentally induced stress and digestion. It should be noted that we deliberately chose a small ACTH dose to simulate physiological stress conditions (Satterlee et al., 1977; Rudolph, 1982).

Faecal samples were collected weekly from each rabbit. After removing accidental soft faeces contamination, the faecal samples were stored at −24 °C until analysed. Chemical examination of the feed and faeces was performed according to the recommendations of the A.O.A.C. (1970). Gross energy content was determined with an adiabatic bomb calorimeter. The faeces minerals were assayed by atom-absorption spectrophotometry (Perkin-Elmer, 1979), colorimetry and turbidimetry. In calculating the digestibility coefficients, the quantity of caecotroph was not taken into consideration (Fekete and Bokori, 1985). Statistical evaluation was carried out as described by Pearce (1965).

Results

The mean digestibility coefficients measured in the 2nd and 3rd week ("physiological state"), in the 4th week ("transition period") and in the 5th and 6th weekof the experiment ("simulated stress: ACTH treatment") are sum-

Table II

Effect of 30 mg/kg virginiamycin given in the feed on the digestibility coefficients (DC) in different periods of the experiment in the control (C) and antibiotic-treated (A) group (mean ± SEM)

Group	Digestibility coefficients (DC), %									
	dry matter		crude ash		organic matter		crude protein		gross energy	
	C	A	C	A	C	A	C	A	C	A
Weeks 2–3	75.7	75.4	48.9*	38.7	77.3	77.3	79.7*	84.5*	75.2	75.4
	1.1	0.9	2.4	2.9	1.1	0.7	1.4	1.1	1.2	0.9
Week 4	76.3	75.2	41.4	32.2	78.3	77.3	80.6	83.5	76.0	75.3
	1.3	2.5	7.5	5.7	1.6	2.4	0.4	2.2	1.4	2.4
Weeks 5–6	76.0	74.9	55.1*	43.6*	77.3	76.3	80.3*	83.7*	75.5	74.5
	0.4	0.4	0.4	3.3	0.5	0.4	0.1	0.8	0.4	0.3
Weeks 2–6	75.9	75.2	49.6**	38.9**	77.4	76.9	80.0**	84.0**	75.4	75.0
	0.5	0.5	1.8	2.1	0.5	0.5	0.6	0.6	0.5	0.5

* $P < 0.01$; ** $P < 0.05$
DC = Digestibility coefficient

Table III

Mineral composition of faecal samples, grouped according to antibiotic supplementation and ACTH treatment (mean ± SEM)

Mineral	Groups and number of samples									
	Virginiamycin-free diet, no ACTH treatment (n = 10)		Virginiamycin-free diet, total (n = 25)		Virginiamycin supplementation (30 mg/kg feed) (n = 25)		No ACTH treatment (n = 20)*		ACTH treatment (n = 20)*	
Potassium, g/kg	4.8±	1.05	5.2±	1.3	4.1±	1.0	4.0±	1.1	5.1±	1.2
Calcium, g/kg	17.6±	0.65	17.8±	2.3	18.0±	2.1	17.9±	1.3	17.2±	2.7
Phosphorus, g/kg	16.2±	1.05	16.3±	2.1	16.0±	2.0	16.3±	1.1	15.2±	2.6
Sulphur, g/kg	3.7±	0.36	3.8±	0.6	3.9±	0.6	3.6±	0.5	3.9±	0.7
Magnesium, g/kg	5.8±	0.39	5.7±	0.6	5.4±	0.7	5.7±	0.7	5.4±	0.9
Sodium, g/kg	1.8±	0.65	1.6±	0.6	2.1±	0.7	2.1±	0.7	1.7±	0.7
Aluminium, mg/kg	182.0±	74.0	165.0±	80.0	213.0±	152.0	218.0±	153.0	181.0±	105.0
Iron, mg/kg	748.0±	160.0	812.0±	234.0	911.0±	239.0	815.0±	150.0	949.0±	295.0
Manganese, mg/kg	215.0±	15.0	209.0±	16.0	210.0±	23.0	213.0±	16.0	211.0±	16.0
Zinc, mg/kg	317.0±	27.0	321.0±	30.0	322.0±	25.0	320.0±	25.0	327.0±	32.0
Copper, mg/kg	55.0±	1.4	55.0±	2.6	66.0±	18.0	60.0±	10.0	57.0±	4.0
Silicon, mg/kg	5.0±	0.4	5.0±	0.6	5.0±	0.6	6.0±	0.5	5.0±	0.7

* Faecal samples collected in the transition period (week 4) are not included

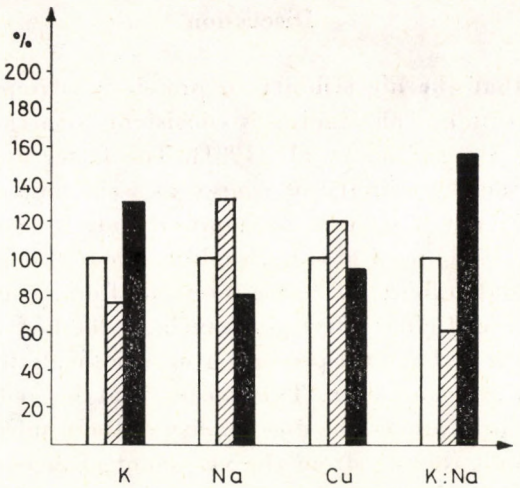


Fig. 1. Influence of virginiamycin (striated columns) and ACTH treatment (black columns) on the mineral content of rabbit faeces (for detailed data see Table III)

marized in Table II. Virginiamycin supplementation of the ration had practically no influence on the digestibility of dry matter, organic matter and gross energy. The digestibility of crude ash, on the other hand, was lower (by 9.2 to 11.5%) throughout the experiment in the rabbits fed the antibiotic-supplemented diet. Virginiamycin feeding definitely improved crude protein digestibility. The inter-group differences were significant ($P < 0.05$ and $P < 0.01$) in all phases of the experiment but in the 4th week.

Considering the entire 6-week experimental period, the daily mean body mass gain of the virginiamycin-fed and control rabbits was 11.75 ± 2.3 and 9.9 ± 4.3 g, respectively, as determined by weekly weighings. The difference is not significant.

The mineral composition of the 50 individual faecal samples (faecal samples collected from 10 rabbits weekly, over a period of 5 weeks) of virginiamycin-treated and ACTH-treated rabbits is shown in Table III. The mineral composition of the 10 faecal samples collected from rabbits receiving neither antibiotic supplementation nor ACTH treatment (control group, 2nd and 3rd weeks) is given for comparison. It can be seen that, although not significantly, virginiamycin decreased faecal K^+ and increased faecal Na^+ and Cu^{2+} concentration. ACTH treatment seemed to exert an opposite effect on the faecal concentration of these minerals. The faecal concentration of Ca, P, S, Mg, Al, Fe, Mn, Zn and Si was altered neither by virginiamycin feeding nor by ACTH treatment.

Discussion

We found that the digestibility of protein was improved by virginiamycin supplementation. This finding is consistent with the results of Rigoni et al. (1980) and Parigi-Bini et al. (1981). The latter authors observed an improvement in the digestibility of energy as well; however, in the present experiment we did not find such an improvement. It should be noted that Parigi-Bini et al. (1981) used growing rabbits, whereas our experiments were conducted with adult rabbits of stable intestinal flora. Vervaeke et al. (1979) attribute the beneficial effect of virginiamycin to the fact that this antibiotic inhibits the growth of bacteria producing organic acids (lactic acid, butyric acid, acetic acid) and ammonia. This means that by reducing protein and carbohydrate loss it improves the digestibility of these substances. Our results suggest that in adult rabbits (from the viewpoint of digestive physiology the rabbits used in the present experiments can be considered adult; Lebas, 1973; Hullár and Gippert, 1986) virginiamycin improves only protein digestibility.

As ACTH treatment failed to alter the digestibility of nutrients, no corrections for possible stress conditions are needed if the animals' requirements are expressed in terms of digestible energy.

For the entire 6-week experimental period the daily average body mass gain of rabbits fed the virginiamycin-supplemented diet exceeded that of the controls by 1.75 g, a difference neither statistically significant nor biologically important. This finding is not surprising since the growth-promoting antibiotics are known to be most effective and economically successful in young animals of high growth rate (Kovács, 1970).

The mineral composition of the faeces was within the ranges reported earlier (Fekete, 1984). For most of the elements Bokori and Tölgyesi (1986) reported similar concentrations; however, they found three or four times higher potassium concentrations than the mean value obtained in the present study. In our opinion this disparity can be explained by the fact that in their case the faecal samples collected under practical conditions might have become contaminated with urine, which is rich in potassium (Wood and Lee, 1985).

Virginiamycin tended to decrease, whereas ACTH treatment tended to increase, the potassium concentration of the faeces, while the faecal concentration of sodium and copper changed in the opposite direction. Upon the effect of virginiamycin the K : Na ratio of the faeces decreased from 3.25 to 1.95 (Fig. 1), suggesting that ACTH treatment may exert an aldosterone-like effect not only on the minerals present in the urine but also on those contained by the faeces. This is physiologically possible as potassium may get into the colon by active transport (Phillips et al., 1966). The effect of virginiamycin is reverse. As opposed to what was reported by Roe (1974) and Henry et al. (1986), calcium and manganese excretion did not change.

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EFFECT OF SALINOMYCIN, FLAVOMYCIN AND AVOPARCIN ON SOME PHYSIOLOGICAL TRAITS OF GROWING LAMBS, WITH PARTICULAR RESPECT TO RUMEN FERMENTATION

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The effects exerted by the growth-promoting antibiotics salinomycin, flavophospholipol (Flavomycin) and avoparcin were studied in experiments conducted with rumen-fistulated wethers. A study was made of how these compounds influenced rumen fermentation and, through the latter, certain blood parameters. Salinomycin was found to affect ruminal carbohydrate metabolism in the first place, by decreasing the molar ratio of acetic acid and by raising that of propionic acid. Under conditions of intensive fattening this effect is of prime importance. Salinomycin feeding resulted in a significant reduction of blood ammonia and total acetic acid concentrations. Flavomycin induced alterations in rumen nitrogen (N) metabolism by reducing ammonia concentration in the rumen. Of the three growth promotants tested, avoparcin resulted in the highest rumen pH values favourable for proteolysis and unfavourable for volatile fatty acid (VFA) production as evidenced by reduced total VFA concentrations.

Keywords: Salinomycin, avoparcin, Flavomycin, growth-promoting antibiotics, lamb, physiology, rumen fermentation.

Of the publications dealing with possible applications of biologically active compounds, works by Chalupa (1977) and, in Hungary, Juhász (1982) gave a detailed description of the applied chemicals and other substances including antibiotics with a growth promoting effect. Antibiotics used in ruminant feeding, among them salinomycin (Sacox®, Hoechst), flavophospholipol (Flavomycin®, Hoechst) and avoparcin (Avoparcin®, American Cyanamid Co.) have been demonstrated to increase body mass gain and to improve feed efficiency when fed at a low dose rate. The international literature contains experimental data for cattle in the first place (Burgstaller et al., 1981; Berger and Fahey, 1983; Hicks et al., 1985; Leitgeb et al., 1985; Barclay et al., 1986). Rather few data are available for sheep (Froetschel et al., 1983; Bedő et al., 1985; Merchen and Berger, 1985; Kobayashi et al., 1986).

The metabolic effects of salinomycin, Flavomycin and avoparcin used as growth promotants were also studied by a number of investigators. However, the results obtained are contradictory in some respects. All three growth promotants are known to affect carbohydrate metabolism in the rumen and to produce significant changes in the energy balance of ruminant animals.

At the same time, they exert an influence on rumen proteolysis, as evidenced by a reduction in ammonia-N concentration. These results were obtained in experiments with beef cattle in the first place. Experiments conducted with sheep have been restricted to mature animals. The objective of the present work was to compare the effects of salinomycin, Flavomycin and avoparcin on rumen fermentation in growing lambs and to determine how these antibiotics influenced rumen fermentation processes and, through the latter, certain blood parameters.

Materials and methods

Merino wether lambs of 18 kg starting body mass were used. Previously the animals had been fitted up, surgically, with Jarret's rumen fistula in the dorsal sac of the rumen. After a wound healing period of 2 weeks, at 18 kg

Table I

Effect of salinomycin on total volatile fatty acid concentration, $C_2 : C_3$ ratio and urea content of the rumen fluid

Time of sampling (hours after feeding)	Group		Total VFA (mmol/l)	$C_2 : C_3$	Urea (mmol/l)
0	Control	$\bar{x}$	56.72	2.13	2.67
		$\pm$SD	10.15	0.28	0.59
	Salinomycin	$\bar{x}$	54.35	1.89	3.58*
		$\pm$SD	5.97	0.24	0.25
1.5	Control	$\bar{x}$	79.16	1.58	2.91
		$\pm$SD	11.54	0.08	0.60
	Salinomycin	$\bar{x}$	94.81	1.37	4.59**
		$\pm$SD	13.03	0.28	0.29
2.5	Control	$\bar{x}$	94.61	1.83	2.87
		$\pm$SD	13.78	0.18	0.75
	Salinomycin	$\bar{x}$	104.31	1.33**	3.35
		$\pm$SD	13.41	0.19	0.22
3.5	Control	$\bar{x}$	98.25	1.96	2.66
		$\pm$SD	10.43	0.07	0.81
	Salinomycin	$\bar{x}$	99.77	1.24***	2.93
		$\pm$SD	4.88	0.12	0.22
5	Control	$\bar{x}$	90.81	1.85	2.53
		$\pm$SD	10.25	0.27	0.87
	Salinomycin	$\bar{x}$	98.30	1.19**	2.62
		$\pm$SD	3.17	0.21	0.24
6.5	Control	$\bar{x}$	84.67	1.83	2.23
		$\pm$SD	7.14	0.18	1.03
	Salinomycin	$\bar{x}$	98.78	1.14**	2.44
		$\pm$SD	10.83	0.21	0.32

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

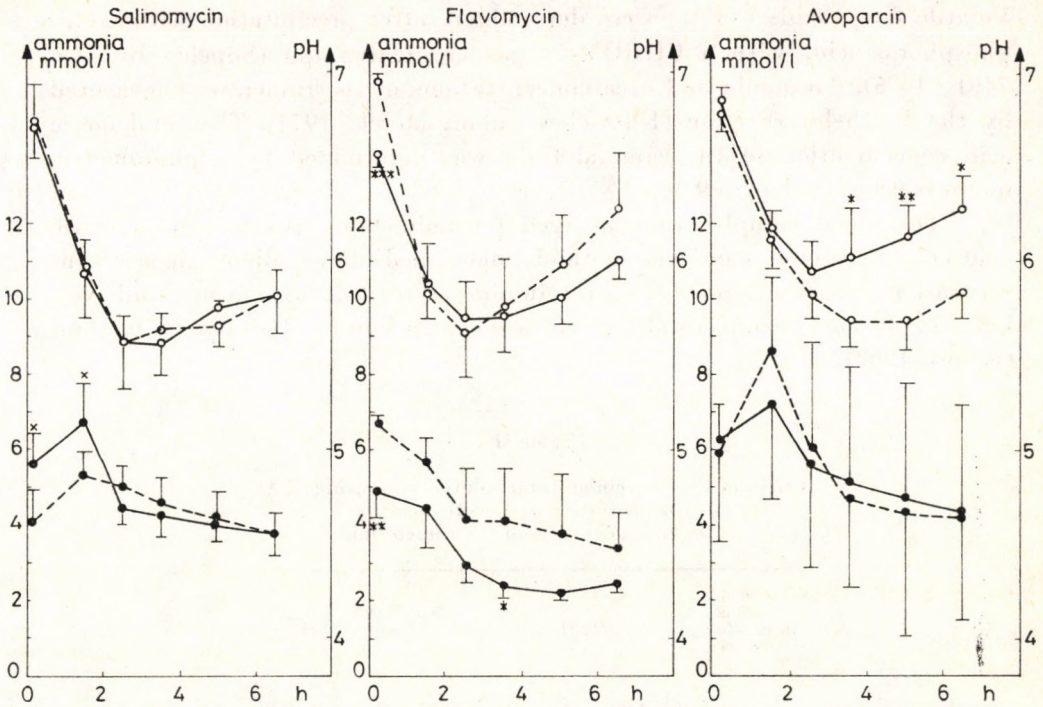


Fig. 1. pH and ammonia concentration changes in the rumen fluid on growth promotant feeding (⊙: pH; ●: ammonia; broken line: control; solid line: growth promotant; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$

body mass the wethers were placed in metabolic cages. Four wethers were used fot each growth promotant. A two-compartment, self-controllcd experiment was carried out: in the first stage the basal diet, and in the second one immediately following the first the diet supplemented with the growth promotant to be tested was fed to the same four wethers. Both phases lasted 21 days. The growth promotants were administered at the following dose rates: salinomycin 20 mg/kg of feed, Flavomycin 15 mg/kg of feed, avoparcin 24 mg/kg of feed. The wethers consumed a ration of 800 g daily (nutritive value: NE_m 7.89 MJ/kg; NE_g 5.23 MJ/kg; digestible crude protein 157.03 g/kg) in two parts, at 8.30 o'clock a.m. and at 15 o'clock p.m. At the morning feeding 100 g meadow hay was offered in addition to the diet. Drinking water was available ad libitum. Sampling took place at the end of the first and the second phase (on day 21) in each experiment. Blood and rumen fluid samples were taken before feeding and 1.5, 2.5, 3.5, 5.0 and 6.5 h after feeding. The pH of the ruminal fluid was measured with a PHM-27 type pH meter (Radiometer, Copenhagen) immediately after each sampling. Ruminal fluid samples were filtered, centrifuged and assayed for various constituents as follows.

Volatile fatty acids (VFAs) were determined, after precipitation with metaphosphoric acid, with a CHROM-41 gas chromatograph (Supelco Bulletin 749E, 1975). Ammonia and urea concentrations of the rumen were measured by the Berthelot reaction (Klinisches Labor. Merck, 1974). The total lactic acid concentration of the ruminal fluid was determined by a photometric micro-method (Velősy, 1979).

The blood samples were assayed for ammonia, urea and total lactic acid concentrations, using the methods mentioned above. Blood glucose concentration was determined using o-toluidine as reagent (Hyvärinen and Nikkilä, 1973) and serum total protein concentration by the biuret method (Bálint, 1962).

Table II

Effect of Flavomycin on total volatile fatty acid (VFA) concentration and acetic acid: propionic acid ratio of the rumen fluid

Time of sampling (hours after feeding)	Group		Total VFA (mmol/l)	$C_2 : C_3$
0	Control	$\bar{x}$	53.31	2.10
		±SD	9.97	0.29
	Flavomycin	$\bar{x}$	70.90*	1.92
		±SD	8.70	0.35
1.5	Control	$\bar{x}$	95.99	1.64
		±SD	16.68	0.23
	Flavomycin	$\bar{x}$	95.83	1.53
		±SD	14.58	0.09
2.5	Control	$\bar{x}$	112.91	1.46
		±SD	19.39	0.24
	Flavomycin	$\bar{x}$	106.39	1.56
		±SD	12.29	0.26
3.5	Control	$\bar{x}$	121.88	1.58
		±SD	10.90	0.10
	Flavomycin	$\bar{x}$	108.86	1.44
		±SD	7.71	0.19
5	Control	$\bar{x}$	99.80	1.61
		±SD	10.16	0.13
	Flavomycin	$\bar{x}$	108.76	1.55
		±SD	6.69	0.24
6.5	Control	$\bar{x}$	91.25	1.82
		±SD	10.16	0.16
	Flavomycin	$\bar{x}$	109.06*	1.58
		±SD	7.46	0.25

* $P < 0.05$

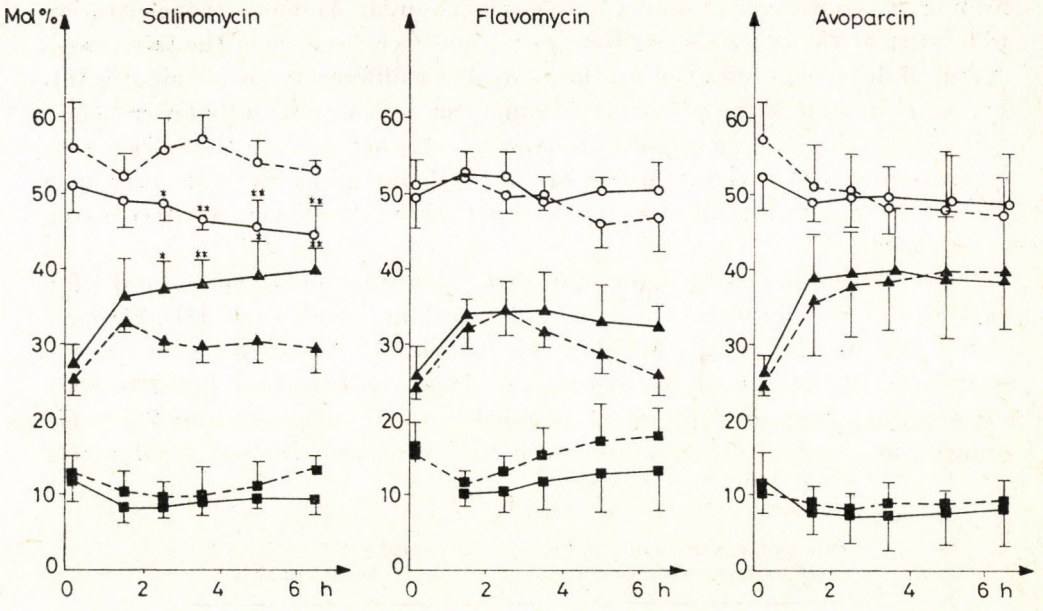


Fig. 2. Effect of growth promotants on the molar % of acetic acid, propionic acid and n-butyric acid (○: acetic acid; ▲: propionic acid; ■: n-butyric acid; broken line: control; solid line: growth promotant; * $P < 0.05$; ** $P < 0.01$

Results

Changes induced in rumen fermentation by the growth promotants tested are shown in Tables I, II and III and in Figs 1 and 2. Results significantly different from the control are shown, together with those referred to in the Discussion. Of the VFAs tested, the percental concentrations of isobutyric acid, isovaleric acid and n-valeric acid are not given, since these compounds failed to induce changes. Similarly, no difference was found between salinomycin- and Flavomycin-treated wethers in total lactate concentration of the rumen and between Flavomycin- and avoparcin-fed animals in ruminal urea contents.

Table I and Fig. 1 indicate that salinomycin feeding failed to alter total VFA concentration and pH of the rumen, as compared to the control. However, at the last four determinations the acetic acid: propionic acid ratio was significantly lower. Before and 1.5 h after feeding ruminal urea and ammonia concentrations of the treated wethers were significantly higher than those of the controls; however, later on no such difference was demonstrable.

In lambs fed Flavomycin the most important change occurred in ammonia concentration of the ruminal fluid (Fig. 1). Before and 3.5 h after

feeding a significantly lower value was measured. Ammonia concentration was lower at the other testing times, too; however, because of the fairly high standard deviations obtained for the control, the difference was not significant. Figure 1 shows that the pH of the ruminal fluid was significantly lower before feeding. At that time a significant increase was observed in total VFA concentration of the rumen. On the other hand, in the acetic acid: propionic acid ratio significant differences were not obtained at any of the testing times (Table II).

Avoparcin produced significant changes in rumen pH and in total VFA and total lactate concentration of the ruminal fluid (Fig. 1, Table III). Figure 1 shows that in the avoparcin-fed group rumen pH was higher than in the control group throughout the experiment. However, avoparcin failed to alter the ammonia concentration of the ruminal fluid. It can be seen in Table III that at the last four examinations total VFA concentration was significantly

Table III

Effect of avoparcin on total volatile fatty acid (VFA) and total lactic acid concentration and $C_2 : C_3$ ratio of the rumen fluid

Time of sampling (hours after feeding)	Group		Total VFA (mmol/l)	$C_2 : C_3$	Total lactic acid (mmol/l)
0	Control	$\bar{x}$	49.01	2.35	1.42
		±SD	12.26	0.16	0.05
	Avoparcin	$\bar{x}$	43.11	2.04	1.32
		±SD	7.25	0.36	0.16
1.5	Control	$\bar{x}$	94.01	1.50	1.77
		±SD	8.97	0.49	0.11
	Avoparcin	$\bar{x}$	80.68	1.30	1.86
		±SD	13.52	0.26	0.50
2.5	Control	$\bar{x}$	111.55	1.40	1.53
		±SD	14.15	0.43	0.12
	Avoparcin	$\bar{x}$	83.11*	1.33	1.35*
		±SD	13.04	0.37	0.18
3.5	Control	$\bar{x}$	117.31	1.31	1.50
		±SD	6.17	0.34	0.04
	Avoparcin	$\bar{x}$	90.77**	1.30	1.32***
		±SD	8.14	0.39	0.03
5	Control	$\bar{x}$	127.61	1.31	1.50
		±SD	19.07	0.60	0.08
	Avoparcin	$\bar{x}$	94.86*	1.34	1.55
		±SD	12.56	0.43	0.18
6.5	Control	$\bar{x}$	119.61	1.26	1.40
		±SD	15.58	0.37	0.09
	Avoparcin	$\bar{x}$	87.30	1.37	1.42
		±SD	10.53	0.53	0.16

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

Table IV

Blood ammonia concentrations in lambs fed different growth promotants

	Time of sampling (h)					
	0	1.5	2.5	3.5	5	6.5
	$\bar{x} \pm SD$					
Control	95.66 ±9.77	81.48 ± 7.74	85.87 ± 9.17	87.77 ±5.35	86.07 ±4.75	81.18 ± 5.62
Salinomycin	74.02* ±7.87	92.60 ±14.83	82.31 ± 8.60	73.82* ±7.11	59.99*** ±6.74	61.17*** ± 2.13
Control	110.56 ±6.67	119.48 ± 9.21	100.15 ±10.25	100.74 ±7.52	98.26 ±6.21	97.27 ±10.44
Flavomycin	97.90* ±3.97	108.24 ± 7.05	100.56 ±10.62	102.73 ±7.34	98.39 ±7.80	98.69 ± 3.41
Control	107.65 ±3.83	96.84 ± 1.36	105.89 ±7.51	100.70 ±2.27	104.40 ±2.05	108.71 ± 1.13
Avoparcin	108.26 ±7.36	109.93 ± 8.07	99.74 ±5.92	105.63 ±6.98	101.14 ±4.93	108.88 ± 1.33

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

lower, and 2.5 and 3.5 h after feeding total lactate concentration of the ruminal fluid was lower than in the control group. In the acetic acid: propionic acid ratio no difference from the control was found at any of the testing times.

The effect of the growth promotants tested on the percental concentration of the three most important VFAs is shown in Fig. 2. In animals fed salinomycin, the molar ratios of acetic acid and propionic acid differed from the control at each testing time, starting with 2.5 h after feeding: the molar ratio of acetic acid increased, while that of propionic acid decreased. Salinomycin did not alter the percental concentration of n-butyric acid. In wether lambs fed Flavomycin or avoparcin, no significant change was demonstrable in the molar ratios of the VFAs tested.

Blood ammonia concentrations are shown in Table IV. Before feeding and at the three last testing times salinomycin-fed animals had significantly lower blood ammonia concentration than the controls. In sheep fed Flavomycin or avoparcin no significant change occurred, except for the pre-feeding value found in animals fed Flavomycin.

Since none of the three growth promotants produced changes in serum urea and total protein concentration, and Flavomycin and avoparcin feeding did not alter blood glucose and total lactic acid concentrations either, these data are not given in tabular form. At the same time, salinomycin did cause a considerable change in blood glucose and total lactic acid concentration (Fig. 3).

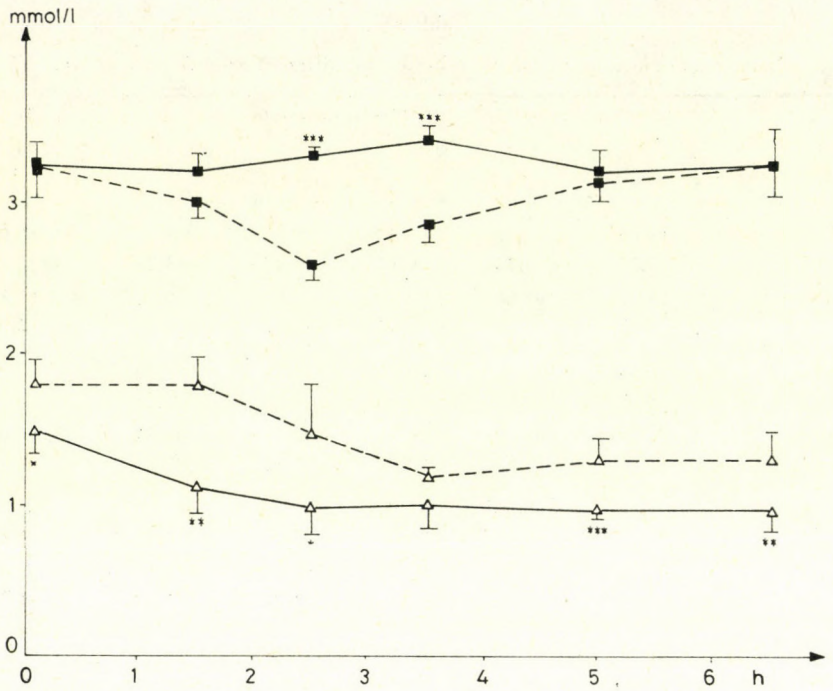


Fig. 3. Effect of salinomycin on blood glucose and total lactic acid concentrations (■: blood glucose; △: blood total lactic acid; broken line: control; solid line: salinomycin; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$

It can be seen that 2.5 and 3.5 h after feeding blood glucose concentration was significantly higher than in the control group, whereas blood total lactic acid concentration was significantly lower throughout the experiment.

Discussion

Of the three growth-promoting antibiotics tested, salinomycin exerted the most pronounced effect on rumen fermentation, by decreasing the molar proportion of acetic acid and raising that of propionic acid, which resulted in a decreased acetic acid: propionic acid ratio. Salinomycin did not alter rumen pH. These results are consistent with those obtained by other researchers (Fontenot et al., 1980; McClure et al., 1980; Merchen and Berger, 1985).

Rumen pH was between 6.7 and 5.6 in both the salinomycin-treated and control group. The onset of fermentation resulted in a pH value of 5.6 which was very favourable to the production of propionic acid. In the salinomycin-fed animals even more propionic acid was produced at pH 5.6 than in the control ones. In growing animals and in those intended for meat production the increased molar proportion of propionic acid is of great importance.

Propionic acid is important also because, as opposed to acetic acid and butyric acid, it leads to H_2 uptake in the rumen, thereby decreasing methane production (methane production deprives the organism of energy). Propionic acid is a unique volatile fatty acid also because it is of glyconeogenetic nature. This role is particularly pronounced in ruminants where small amounts of glucose are absorbed from the digestive tract and, correspondingly, glyconeogenesis is essential. Salinomycin brought about a rise in propionic acid production; as a result, also more glucose must have been produced. This is a possible explanation for the increase found in blood glucose concentration. However, in this rise lactic acid, which also has a glyconeogenetic property, might also have played a role, since blood total lactic acid concentration decreased upon salinomycin feeding (Fig. 3).

As regards N metabolism of the rumen, urea and ammonia concentration did not change (with the exception of samples taken before and 90 min after feeding). McClure et al. (1980) reported similar results. On the other hand, according to Piva et al. (1981) salinomycin raised ammonia concentration in the rumen, whereas Webb et al. (1980) observed a reduction in ruminal ammonia-N after salinomycin feeding.

In our experiment, salinomycin feeding decreased rumen pH below 6.0. At such pH values the degree of protein hydrolysis must have decreased, since the pH optimum of proteolysis is between 6.5 and 7.0. The fact that blood ammonia concentration was significantly lower after salinomycin feeding means that the ammonia derived from reduced proteolysis had been utilized in the rumen, and less ammonia had been absorbed.

In accordance with Galbraith et al. (1983), we failed to demonstrate changes in serum urea concentration.

Flavomycin exerted a different effect on rumen fermentation than salinomycin: it did not alter the molar proportions and ratio of acetic acid and propionic acid as compared to the control. After Flavomycin feeding the molar proportion of n-butyric acid was lower (but not significantly) than in the control group. This was favourable since from this smaller amount of butyric acid less gas, primarily less methane, was produced.

These results are consistent with data reported by Burgstaller et al. (1981) and Galbraith et al. (1983). It should be noted, however, that others found significant alterations in rumen fermentation processes. In the opinion of Piva et al. (1981), Flavomycin increased the concentration of acetic acid and butyric acid and reduced that of propionic acid. While in the Flavomycin-fed group rumen pH ranged between 6.56 and 5.68 and by the end of the experiment it had reached 6.03, in the control group pH values between 6.88 and 5.61 were found and at the end of the experiment pH was 6.28. Thus, in the Flavomycin-fed group the pH range was narrower and no major shifts above 6.0 occurred. These two factors reduced the degree of proteolysis and

deamination. The lower rumen ammonia concentration observed after Flavomycin feeding might have been consequent upon reduced proteolysis. Another possible cause may be that the resorption rate of ammonia exceeded the rate of its production. As a result, ammonia concentration did not change in the blood but decreased in the rumen.

Avoparcin feeding resulted in a by 7% higher rumen pH and a by 25% lower VFA concentration, on average, at the last three measurement times. Considering all the measurements, in avoparcin-fed lambs rumen pH ranged between 5.95 and 6.87 and at the end of the experiment it was 6.27. Thus, of the three growth promotants tested, avoparcin resulted in the highest rumen pH. In the avoparcin-fed group rumen pH was above 6.0 in all cases but one. Such pH values are not too favourable for the production of VFA and lactic acid. This is why we measured significantly lower total VFA and total lactic acid concentrations. Although total VFA concentration decreased, the molar ratios of VFAs were not different from the control values. Neither was there a difference in the acetic acid : propionic acid ratio; the values were nearly identical with those obtained for the control sheep.

The pH range induced in the rumen by avoparcin feeding was favourable for proteolysis which, in turn, yielded more ammonia. The produced ammonia was partly absorbed, partly incorporated into the microorganisms. Since neither blood nor rumen ammonia concentration changed as compared to the control, the production, utilization and absorption of ammonia must have been in a state of equilibrium.

Dissenting views have been published in the special literature on the effect exerted by avoparcin on rumen fermentation. Several authors (Ingle et al., 1978; Chalupa et al., 1981; Cottyn et al., 1983; Froetschel et al., 1983) reported that after avoparcin feeding rumen pH and the molar proportion of propionic acid rose, whereas that of acetic acid decreased. Froetschel et al. (1983) found a decrease in total VFA concentration, too. In their opinion, avoparcin exerts its effect by decreasing the decomposition of proteins or amino acids. Others (Dyer et al., 1980; Darden et al., 1985; Unsworth et al., 1985) failed to observe differences in the concentration of the various VFAs. Darden et al. (1985) found no alteration in ammonia concentration after avoparcin feeding.

In summary, we can establish that the growth promotants tested exert disparate effects on rumen fermentation processes and proteolysis.

Salinomycin had a beneficial effect on carbohydrate metabolism by enhancing propionic fermentation in the rumen. This permitted better utilization of the feed energy since the energy loss caused by methane and carbon dioxide production decreased. A further advantage is that propionic acid can change into carbohydrate in the organism and, thus, it disburdens the amino acids in glyconeogenesis.

Flavomycin had an influence on ruminal nitrogen metabolism by reducing ammonia concentration.

Of the three growth-promoting antibiotics avoparcin resulted in the highest rumen pH and the lowest total VFA concentration.

The present experiments have revealed that there must be a correlation between the active substance and the feed acting as substrate. Namely, all three active substances, given in the same feed, exerted different effect on fermentation and proteolysis. If the physical and chemical composition of a given feed is suited to the ruminant animal, after adding the growth promotant it surely will not remain so. This can be an explanation for the contradictory results obtained by various researchers in numerous experiments. For each active substance an optimal protein and energy level should be adjusted at which they have maximum efficacy. Thus, all three growth promotants fed in the present experiments could be supplemented with NPN compounds which would meet the ammonia requirements of microorganisms.

In the case of Flavomycin and avoparcin, optimal efficacy of the active substance could be facilitated by giving larger amounts of carbohydrates which are easy to decompose. On the one hand, this would provide the microorganisms with the energy needed to be able to incorporate ammonia produced during the decomposition of feed proteins and/or NPN compounds; on the other, it would have a beneficial effect on the host's energy balance. These statements may serve as a basis for further experiments.

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PHYSIOLOGICAL EFFECTS OF HALOTHANE ANAESTHESIA UNDER SPONTANEOUS BREATHING IN CALVES

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Six clinically healthy, crossbred male calves (*Bos taurus* L.), 12–15 months old, were used to study the effects of halothane anaesthesia on the cardiovascular dynamics, acid-base status, blood gas tensions and various biochemical constituents of the body. Significant pulmonary hypertension, tachycardia and respiratory acidosis were noticed following halothane anaesthesia. The mean arterial blood pressure, central venous pressure, electrocardiogram and the plasma Na^+, K^+ and Cl^- did not change appreciably.

Keywords: Halothane anaesthesia, calf, cardiovascular dynamics, acid-base status, biochemical constituents.

Anaesthesia in cattle requires specific considerations as there is a need to obtain rapid arousal to avoid rumen bloat, regurgitation and aspiration of the ruminal contents. To moderate such problems, inhalation anaesthetics applied in closed/semi-closed circuit are generally preferred. Among the various inhalation anaesthetics, halothane has been observed to have minimal intra- and post-operative complications in cattle (Adetunji et al., 1984). Although this agent is being used clinically in cattle to maintain anaesthesia, its detailed effects on various body systems have not been worked out. The present study reports changes in haemodynamics, acid-base status and blood gas tensions following halothane anaesthesia in this species.

Materials and methods

The experiments were conducted on six clinically healthy male crossbred calves, 12–15 months old and of 70–100 kg body mass.

All the animals were kept off feed and water 24 h prior to experimentation. The carotid artery and the jugular vein were exteriorized aseptically under local infiltration anaesthesia using 2% procaine HCl. Siliconized catheters filled with heparinized saline were implanted in the carotid artery, right atrium and the pulmonary artery. The position of the catheters in the right atrium and pulmonary artery was confirmed by observing pressure changes in the saline manometer.

Experimental procedure. The animal was restrained in lateral recumbency. The rumen was trocarized to avoid any bloat.

Anaesthesia was induced with 5% thiopental sodium given at the rate of 10 mg/kg body mass. The animal was intubated. The endotracheal tube was connected to a large-animal anaesthetic apparatus (Narkovet, North American Drager, U.S.A.) so as to breathe halothane spontaneously in a closed circuit. The halothane vaporizer was set at a concentration of 3–4% to achieve surgical anaesthesia. The animal was maintained in the surgical plane of anaesthesia for 30 min after the administration of halothane. Blood sampling intervals and recording of the parameters were as follow:

On the day of the experiment, the connecting ends of the catheters were exposed under local anaesthesia. Before induction of anaesthesia, haemodynamics, acid-base, blood gas and other measurements were taken following a 1-h stabilization period in all the animals to form the base values. After induction of anaesthesia with 5% thiopental sodium, the animal was allowed to breathe halothane spontaneously in a semi-closed circuit for 30 min and the blood samples/readings were taken thereafter.

The parameters recorded were:

(A) Haemodynamics:

1) Mean carotid arterial blood pressure (MAP) was recorded with a mercury manometer.
2) Pulmonary artery pressure (PAP) was recorded with a saline manometer.
3) Central venous pressure (CVP) was recorded with a saline manometer.
4) Base-apex electrocardiogram (ECG) was recorded through a transistorized electrocardiograph (Cardiart, British Physical Laboratories, Hyderabad, India) at a paper speed of 25 mm/sec and calibration setting of 1 mV/cm (Norr, 1913). The ECG was analyzed for duration and amplitude of P and T waves and the mean QRS complex, PR interval and segment, QT interval and ST segment.
5) The heart rate (HR) was calculated from the base-apex ECG.

(B) Biochemical parameters:

1) The arterial pH (pHa), partial pressure of carbon dioxide and partial pressure of oxygen of arterial ($paCO_2$ and paO_2) and mixed venous ($p\bar{v}\,CO_2$ and $p\bar{v}\,O_2$) blood were measured by a blood gas analyzer (BME 33 blood micro-equipment, Radiometer, Copenhagen, Denmark).

2) Arterial and venous oxyhaemoglobin was measured with an Ames Blood Analyzer (Miles Laboratories, Australia).
3) Arterial bicarbonate and base excess were derived from Siggaard-Andersen's nomogram using measured pH and pCO_2.
4) Plasma sodium (Na^+) and potassium (K^+) were determined with a flame photometer (Elico Flame Photometer, Hyderabad, India).
5) Plasma chloride (Cl^-) was determined by the method of Schales and Schales (1941).
6) True glucose was measured with an Ames Analyzer.

Statistical analysis of the data. The data were analyzed using one-way analysis of variance followed by a critical difference test. A probability level of $P < 0.05$ was considered statistically significant.

Results and discussion

The mean $\pm$ SEM of various parameters is presented in Table I.

Table I

Mean $\pm$ SEM of various parameters following halothane anaesthesia in calves

Parameter	Base value	During anaesthesia
A. Haemodynamics		
MAP (mm Hg)	124.9 $\pm$ 8.5	123.4 $\pm$ 6.5
PAP (cm of H_2O)	16.6 $\pm$ 2.2	29.8 $\pm$ 3.0*
CVP (cm of H_2O)	4.2 $\pm$ 2.1	3.8 $\pm$ 2.8
HR (per min)	54.9 $\pm$ 4.9	93.7 $\pm$ 8.8*
B. Blood		
pHa	7.450 $\pm$ 0.009	7.338 $\pm$ 0.023*
$paCO_2$ (kPa)	4.44 $\pm$ 0.22	6.62 $\pm$ 0.79*
paO_2 (kPa)	11.4 $\pm$ 0.37	20.64 $\pm$ 2.23*
$p\bar{v}O_2$ (kPa)	3.84 $\pm$ 0.21	5.19 $\pm$ 0.48*
$p\bar{v}CO_2$ (kPa)	5.19 $\pm$ 0.26	7.02 $\pm$ 0.82*
arterial oxyhaemoglobin (g %)	11.8 $\pm$ 0.4	11.9 $\pm$ 0.4
venous oxyhaemoglobin (g %)	11.5 $\pm$ 0.4	11.7 $\pm$ 0.5
arterial HCO_3^- (mmol/l)	23.5 $\pm$ 1.5	25.1 $\pm$ 2.0
arterial BE (mmol/l)	0.33 $\pm$ 1.35	-0.67 $\pm$ 1.73
plasma Na^+ (mmol/l)	125.3 $\pm$ 3.0	125.6 $\pm$ 3.5
plasma K^+ (mmol/l)	4.8 $\pm$ 0.5	4.9 $\pm$ 0.8
plasma Cl^- (mmol/l)	100.8 $\pm$ 2.7	99.0 $\pm$ 0.7
true glucose (mmol/l)	4.96 $\pm$ 0.47	4.86 $\pm$ 0.27

* $P < 0.05$

There was significant pulmonary hypertension, tachycardia and respiratory acidosis during halothane anaesthesia in this investigation. There was no change in MAP.

During halothane anesthesia, tidal volume is decreased and respiration is increased but still the overall effect is a decrease in alveolar ventilation resulting in respiratory acidosis (Gaensler, 1962). Hypercapnia does occur in various species under halothane anaesthesia with SB (Adetunji et al., 1984; Bahlman et al., 1972; Becker and Waelti, 1980; Chandrasakhi, 1982; Gates, 1970; Gates et al., 1971). Many human anaesthetists show a considerable tolerance of hypercapnia which they allow their patients to develop and a $paCO_2$ within a range of 44 to 75 mm Hg (mm Hg $\times$ 0.133 = kPa) is common during anaesthesia (Nunn, 1964; Nunn, 1969). However, hypercapnia causes an increase in plasma catecholamines in man (Nunn, 1964), thereby increasing the MAP (Rose et al., 1983), but at very high levels of $paCO_2$ (above 100 mm Hg) MAP falls and is considered a cause of death if supercarbia persists for an hour or so (Graham et al., 1960). It is postulated that sympathetic efferent activity may help in the maintenance of MAP through CO_2 retention during halothane anaesthesia in the horse (Eberly et al., 1968). However, in man, dog and cat halothane induces hypotension without a compensatory tachycardia, indicating some impairment of the cardiovascular homeostatic mechanism such as baro-reflex function (Ahlgren et al., 1978; Egawa, 1975; Sandford and Colby, 1984; Smith et al., 1974). Even at normocarbic levels, halothane causes hypotension in man and dog (Greene, 1968). In man, at a concentration of 1.8% and above, the ventricular compliance is increased along with the rise in CVP. The increased CVP and the marked reduction in stroke fraction are probably due to the decrease in the contractile performance of the myocardium (Greene, 1968). A high level of hypercapnic acidosis under halothane anaesthesia in man can also result in cardiac arrhythmias (Black et al., 1959). The present investigation did not show any such arrhythmias, probably because the levels of $paCO_2$ were not too high.

The increase in PAP in this study is also in contrast to its unchanged level during halothane anaesthesia in man under SB (Wyant, 1958). However, ether, cyclopropane and barbiturate anaesthesia cause an increase in PAP due to elevated pulmonary vascular resistance or left atrial pressure or hypoxic pulmonary vasoconstriction in man (Nunn, 1969). The possibility of hypoxic vasoconstriction is ruled out in the present case because of the high paO_2 observed in this study. Hypercapnia also causes a rise in pulmonary vascular resistance, along with active pulmonary vasoconstriction. The effects of hypercapnic acidosis on the pulmonary circulation have been assessed under various conditions (Fishman, 1961; Fishman, 1962). There is a prevailing opinion that acidosis accounts for the rise in PAP, and the pulmonary blood flow may remain unchanged or slightly increased (Rudolph and Yuan, 1966). This means

that acidosis would exert the same effect as hypoxia does: a vasoconstriction in the pulmonary arterial bed. Daum et al. (1969) reported an increase in PAP and left atrial pressure depending upon the degree of hypercapnic acidosis in man. It was hypothesized that hypercapnia increases PAP, left atrial pressure and pulmonary wedge pressure due to altered contractility of the myocardium, which still remains to be proven.

Another interesting feature of this investigation has been the tachycardia, in contrast to bradycardia or unaltered HR under halothane anaesthesia in man and cat (Duke et al., 1977; Sandford and Colby, 1984). Halothane depresses the baro-reflex control of HR, thereby causing bradycardia or unaltered HR. However, in the present study there might have been stimulation of the sympathetic nervous system or inhibition of parasympathetic nervous activity and, hence, increased adrenaline mobilization (Price and Onishi, 1980). The traditional concept of complementary sympathetic nervous system activity and parasympathetic nervous system activity mediating the arterial baroreceptor reflex control of the HR (Heymans and Neil, 1958) has been shown to be incomplete and species-dependent (Hamlin et al., 1972; Pickering et al., 1972; Scher et al., 1972; Rothbaum et al., 1974). Predominantly parasympathetic nervous system activity is responsible for the response to increasing MAP in the horse and cattle (Slinker et al., 1982) and predominantly sympathetic nervous system activity is responsible for the response to decreasing MAP in cattle. Hence, this tachycardia might also be responsible for the maintained MAP in this investigation.

In summary, this study indicates that the effects of halothane on various cardiovascular dynamics are quite moderate. However, keeping in view the hypercapnia, this agent may be used with artificial ventilation in animals with existing cardiopulmonary diseases. Similar recommendations have also been made by Becker and Waelti (1980) following halothane–nitrous oxide anaesthesia in cows.

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STUDIES ON GLUTAMIC-OXALOACETIC AND GLUTAMIC-PYRUVIC TRANSAMINASES FROM MIXED RUMEN BACTERIAL FLORA OF BUFFALOES AND CATTLE

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Glutamic-oxaloacetic (GOT) and glutamic-pyruvic transaminases (GPT) were characterized in the mixed rumen bacteria from buffaloes *(Bubalus bubalis)* and cattle. The pH and temperature optima of both enzymes were 7.4 and 37 °C. These enzymes from both ruminant species were thermostable up to 40 °C and completely inactivated at 80 °C. The optimal concentrations of all the substrates were determined and the Michaelis-Menten constant (K_m) and V_{max} values calculated. The K_m values of both the enzymes for almost all the substrates used were apparently lower in buffaloes than in cattle. The specific activity of GPT was higher than that of GOT in both species. Of the electrolytes tested, $MgCl_2$ (20 mmol/l) and $MnCl_2$ (4 mmol/l) increased GOT activity by 88 and 290%, respectively, while $MgCl_2$ (20 mmol/l), $CaCl_2$ (10 mmol/l) and $MnCl_2$ (4 mmol/l) increased GPT activity by 62, 38 and 360%, respectively, in both species as compared to control conditions. Other chemicals (mmol/l) such as EDTA (10), ammonium molybdate (10), sodium citrate (4), sodium arsenate (1), sodium azide (1) and p-chloro-mercuribenzoate (PCMB) (1) reduced the activity of GOT and GPT by 62 and 57, 100 and 100, 72 and 85, 36 and 20, 47 and 27, 62 and 82%, respectively.

Keywords: Glutamic-oxaloacetic transaminase (GOT), glutamic-pyruvic transaminase (GPT), rumen bacteria, ruminants, buffalo, cattle.

Several enzymes serving for the distribution of amino nitrogen in rumen microorganisms have been demonstrated. Glutamic-oxaloacetic transaminase (GOT) and glutamic-pyruvic transaminase (GPT) are the two most important enzymes of nitrogen metabolism in the rumen microorganisms (Chalupa et al., 1970; Arthur and Bloomfield, 1971). Despite this fact, they have not been fully characterized yet.

In this paper the characterization of GOT and GPT from rumen bacteria of buffaloes and cattle is reported.

Materials and methods

Two rumen-fistulated buffalo bulls *(Bubalus bubalis)* were fed a maintenance ration of 6 kg wheat straw, 2 kg green lucerne, 0.5 kg mustard cake and 0.5 kg crushed maize grains per day. Similarly, two rumen-fistulated bulls

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(Bos taurus) were fed daily 4 kg wheat straw, 2 kg green lucerne, 0.5 kg mustard cake and 0.5 kg crushed maize grains. The animals were maintained on this diet for 3 months.

In the morning before feeding and 4 h after feeding, one litre of rumen fluid (RF) was obtained by suction through a perforated plastic tube from various parts of the rumen. The RF from the two animals each and from the two times of sampling was mixed on an equal volume basis, strained through two layers of cheese cloth and was immediately cooled in an ice bath. The crude enzyme extract was prepared (Baldwin and Palmquist, 1965) from one litre of cold RF and stored at 0 °C in small vials (0.5 ml). The enzyme preparation, termed mixed rumen bacterial extract (MRBE), was stable for several days when kept at 0 °C.

The GOT (EC 2.6.1.1) and GPT (EC 2.6.1.2) activity in MRBE was determined by a colorimetric method as described in Sigma Technical Bulletin No. 505 (Sigma Chemical Co., U.S.A.). Appropriate controls were always included in the assay to estimate the non-enzymatic activity and this was subtracted from the experimental values. The specific activities were expressed as nmol of product formed min^{-1} mg^{-1} protein. The protein content in MRBE was determined by the method of Lowry et al. (1951). The values of the Michaelis-Menten constant (K_m) were determined by conventional methods (Dixon and Webb, 1964) and the values of Q10 and energy of activation were calculated (Segel, 1976).

Results

The GOT from both buffaloes and cattle has a pH optimum of 7.4. In MRBE from buffaloes the activity of GOT was reduced by 20.9, 31.6, 26.3 and 42.1% at pH 6.8, 6.4, 8.0 and 9.0, respectively. The corresponding reduction in cattle was 17.6, 25.5, 41.7 and 58.8%. The temperature optimum of the enzyme in MRBE from both buffaloes and cattle was 37 °C. The energy of activation (Ea) and Q10 of this enzyme between 30 and 40 °C calculated from Arrhenius plots were 35,037 J mol^{-1} and 1.48, respectively, in MRBE from buffaloes and the corresponding values in cows were 33,228 J mol^{-1} and 1.38 (Fig. 1). The thermostability of the enzyme was investigated between 25 and 80 °C. The enzyme preparation (0.5 ml) in 0.05 M phosphate buffer (pH 7.4) was kept at 25, 30, 40, 50, 60, 70 and 80 °C for 15 min. The enzyme was immediately cooled and the residual activity was assayed at 30 °C. The enzyme was found to be stable up to 40 °C in both ruminant species and was completely inactivated at 80 °C. The optimal concentrations (mmol/l) of L-aspartate were 32 and 32, while those of 2-oxoglutarate were 2 and 3 mmol/l in the preparations from buffaloes and cattle, respectively (Table I). The K_m values in buffaloes and cattle for these substrates at pH 7.4 are given in

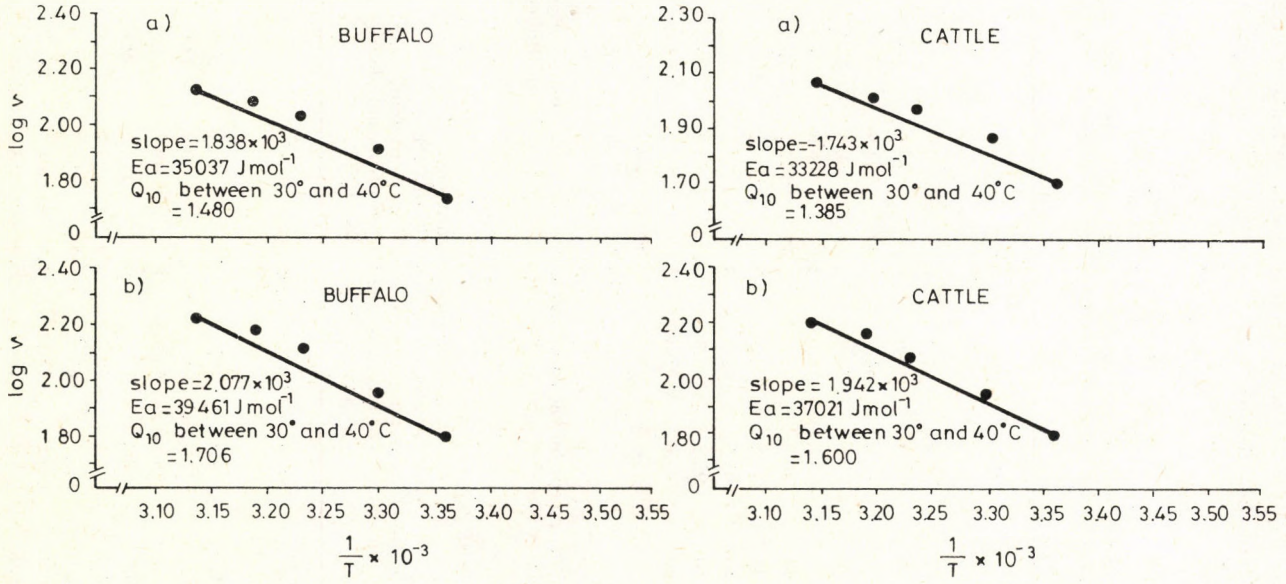


Fig. 1. Arrhenius plot for glutamic-oxaloacetic transaminase (a) and glutamic-pyruvic transaminase (b) from mixed rumen bacterial extract of buffaloes and cattle

Table III. The enzyme activity was not inhibited when the concentration of L-aspartate was increased beyond its optimal level in both ruminant species. However, the activity was reduced when the concentration of 2-oxoglutarate was increased beyond its optimal level.

Table I

Effect of different concentrations of substrates on the activity of GOT (nmol oxaloacetate produced min^{-1} mg^{-1} protein) in extracts of mixed rumen bacteria from buffaloes and cattle (n = 2)

L-aspartate (mmol/l)	Buffalo	Cattle	2-oxo-glutarate (mmol/l)	Buffalo	Cattle
1	35.9	37.7	0.05	29.9	21.5
2	59.8	53.9	0.10	38.9	26.9
4	71.8	64.6	0.20	53.9	32.3
8	77.8	70.0	0.50	68.8	43.1
16	83.8	80.8	1.00	86.8	59.2
24	89.8	91.6	2.00	107.7	75.4
32	95.8	102.3	3.00	89.8	102.3
40	95.8	102.3	4.00	77.8	86.2
60	95.8	102.3	6.00	53.9	80.8
140	95.8	102.3	8.00	41.9	64.6
			12.00	29.9	53.9
			16.00	23.9	43.1

In Tables I and II, except for the substrate whose concentration is varied, the concentration of all other substrates was the same as described in Sigma Technical Bulletin No. 505

Table II

Effect of different concentrations of substrates on the activity of GPT (nmol pyruvate produced min^{-1} mg^{-1} protein) in the extracts of mixed rumen bacteria from buffaloes and cattle (n = 2)

L-alanine (mmol/l)	Buffalo	Cattle	2-oxo-glutarate (mmol/l)	Buffalo	Cattle
1.25	19.9	18.0	0.1	19.9	26.9
2.5	44.9	26.9	0.2	39.9	53.9
5.0	59.8	44.9	0.5	69.8	71.8
10.0	69.8	71.8	1.0	114.7	89.8
20.0	89.8	98.7	2.0	129.7	116.7
40.0	109.7	125.7	3.0	109.7	161.6
50.0	119.7	143.6	4.0	94.8	125.7
60.0	127.2	170.6	6.0	74.8	80.8
80.0	127.2	170.6	8.0	34.9	62.8
120.0	127.2	170.6	10.0	24.9	53.9

Table III

K_m and V_{max} values (determined by Lineweaver-Burk plots) of GOT and GPT in extracts of mixed rumen bacteria from buffaloes and cattle (n = 2; mean ± SD)

Enzyme	Substrate	K_m (mmol/l)		V_{max} (nmol product formed min^{-1} mg^{-1} protein)	
		Buffalo	Cattle	Buffalo	Cattle
GOT	L-aspartate	4.00 ± 0.45	5.00 ± 0.60	102.04 ± 6.01	100.00 ± 7.22
	2-oxoglutarate	0.17 ± 0.006	0.31 ± 0.002	86.95 ± 4.43	84.75 ± 3.87
GPT	L-alanine	8.00 ± 0.88	14.29 ± 1.01	133.33 ± 10.11	166.67 ± 7.63
	2-oxoglutarate	0.67 ± 0.10	0.45 ± 0.03	166.67 ± 4.85	142.86 ± 5.14

Table IV

Effects of different electrolytes on the activity of GOT and GPT in the extracts of mixed rumen bacteria from buffaloes and cattle (n = 2)

Electrolyte (mmol/l)	GOT		GPT		Electrolyte (mmol/l)	GOT		GPT	
	Buffalo	Cattle	Buffalo	Cattle		Buffalo	Cattle	Buffalo	Cattle
$MgCl_2$					$NiCl_2$				
5	104	112	140	145	1	60	60	90	95
10	150	152	150	156	$ZnSO_4$				
20	192	185	160	165	1	84	80	94	96
$CaCl_4$					$CuSO_4$				
2	100	100	108	105	1	53	57	68	70
5	100	100	125	120	$CdSO_4$				
10	100	100	140	136	1	50	54	100	100
$MnCl_2$					$LiSO_4$				
1	120	140	140	155	1	60	60	23	22
2	220	220	300	310	$HgCl_2$				
4	380	400	450	470	1	21	25	41	39
$CoCl_2$					$AgNO_3$				
1	60	63	100	100	1	29	30	38	40

Figures are adjusted by taking the value of the control (without electrolyte) as 100

GPT has pH optima of 7.4 and 7.6 in MRBE from buffaloes and cattle, respectively. In MRBE from buffaloes at pH values of 7.2, 6.4, 8.0 and 9.0 the activities were reduced by 7.5, 30.2, 20.7 and 34.0%, respectively. The corresponding reductions in preparations from cattle were 25.7, 45.7, 31.4 and 54.3%. The temperature optimum of the enzyme in MRBE from both buffaloes and cattle was 37 °C. The Ea and Q10 of this enzyme between 30 and 40 °C were 39,461 J mol^{-1} and 1.71, respectively, in MRBE from buffaloes and the

Table V

Effect of some chemicals on the activity of GOT and GPT in the extracts of mixed rumen bacteria from buffaloes and cattle (n = 2)

Chemical (mmol/l)	GOT		GPT		Chemical (mmol/l)	GOT		GPT	
	Buffalo	Cattle	Buffalo	Cattle		Buffalo	Cattle	Buffalo	Cattle
EDTA					Sodium arsenate				
1	68	66	70	74	1	62	65	80	80
2	56	52	65	67	Sodium azide				
5	45	42	60	62	1	55	50	80	76
Ammonium molybdate					p-chloro-mercuri-benzoate (PCMB)				
1	62	65	80	85	1	36	40	16	20
2	42	44	75	70					
5	40	36	50	48					
10	0	0	0	0					
Sodium citrate									
1	50	50	70	69					
2	42	44	70	67					
4	25	30	15	14					

Figures are adjusted by taking the value of control (without chemical) as 100

corresponding values in cattle were 37,021 J mol^{-1} and 1.60 (Fig. 1). The thermostability of GPT, as measured in the case of GOT (in the case of cattle, pH was, however, 7.6) was 40 °C in both ruminant species. The enzyme was completely inactivated at 80 °C. The optimal concentrations (mmol/l) of L-alanine were 60 and 60, while those of 2-oxoglutarate were 2 and 3 in MRBE from buffaloes and cattle, respectively (Table II). The K_m values for these substrates are given in Table III. Increasing the concentration of L-alanine beyond its optimal level did not inhibit the enzyme activity in either ruminant species, whereas raising the concentration of 2-oxoglutarate beyond its optimal level decreased the reaction velocity.

The specific activity of GOT (under standard assay conditions) was 107.7 and 102.3 in the preparations from buffaloes and cattle, respectively. The corresponding values for GPT were 129.7 and 170.6. It is interesting to note that K_m values of both GOT and GPT for all the substrates used (Table III) were lower in MRBE from buffaloes than in that from cattle except that of GPT for 2-oxoglutarate.

$MgCl_2$ and $MnCl_2$ were found to be the activators of GOT (Table IV). Rising concentrations (mmol/l) of $MgCl_2$ from 5 to 20 and of $MnCl_2$ from 1 to 4 increased the enzyme activity in both buffaloes and cattle. At a $MgCl_2$ concentration of 20 mmol/l and a $MnCl_2$ concentration of 4 mmol/l the enzyme activity was increased by 85–92 ($P < 0.01$) and 280–300% ($P < 0.01$) over

the control values, respectively. $MgCl_2$, $CaCl_2$ and $MnCl_2$ were the activators of GPT from both ruminant species. $MgCl_2$ (20 mmol/l), $CaCl_2$ (10 mmol/l) and $MnCl_2$ (4 mmol/l) increased the enzyme activity by 60–65% ($P < 0.01$), 36–40% ($P < 0.05$) and 350–370% ($P < 0.01$), respectively, over the control values (Table IV). In the case of both GOT and GPT, NaCl (5–50 mmol/l), KCl (5–50 mmol/l) and $(NH_4)_3PO_4$ (50 mmol/l) were without effect. Other salts were inhibitory. The effect of various chemicals on the activity of GOT and GPT were also tested (Table V). EDTA (10 mmol/l), ammonium molybdate (10 mmol/l), sodium citrate (4 mmol/l), sodium arsenate (1 mmol/l), sodium azide (1 mmol/l) and PCMB (1 mmol/l) reduced the activity of GOT and GPT by 60–65 and 55–60, 100 and 100, 70–75 and 85, 35–38 and 20, 45–50 and 20–34, 60–64 and 80–84%, respectively, in both ruminant species. Pyridoxine hydrochloride (2–20 mmol/l) and sodium glutamate (2–20 mmol/l) were without effect.

Discussion

The presence of keto acids like alpha-ketoglutaric acid and pyruvic acid (Van der Horst, 1961) and free amino acids (Write and Hungate, 1967) in the rumen suggests the involvement of transaminase reactions in amino acid biosynthesis. The data on the specific activity values of GOT and GPT in this study, and of glutamate dehydrogenase in an earlier work (Saini and Singh, 1987) indicated that transamination reactions are also important in the nitrogen metabolism of rumen microorganisms. The specific activity of GPT was apparently higher than that of GOT in MRBE from both ruminant species. Based on the high activities of both GOT and GPT in the mixed rumen bacteria from sheep, Chalupa et al. (1970) also indicated the importance of transamination reactions in the assimilation of ammonia into cellular proteins by rumen bacteria. Similar views were expressed by Arthur and Bloomfield (1971). The activity of GOT in the extracts of rumen microorganisms from cows (Baldwin and Palmquist, 1965; Erfle et al., 1977) and of both GOT and GPT in sheep (Wallace, 1979) and buffaloes (Bhatia et al., 1979) was also detected earlier.

The results concerning the effect of various electrolytes (Table IV) and chemicals (Table V) on the activities of GOT and GPT indicate that the activities of these enzymes in the rumen can be manipulated.

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STUDIES ON GLUTAMINE AND ASPARAGINE SYNTHETASES FROM MIXED RUMEN BACTERIAL FLORA OF BUFFALOES AND CATTLE

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Glutamine (GS) and asparagine synthetases (AS) have been characterized in the mixed rumen bacterial flora from buffaloes *(Bubalus bubalis)* and cattle *(Bos taurus)*. The pH optima of GS was 7.6 in both ruminant species and that of AS was 7.2 and 7.0 in buffaloes and cattle, respectively. The optimal concentrations of all the substrates were determined and the K_m and V_{max} values calculated. The K_m and V_{max} values of both enzymes for all the substrates used, except sodium glutamate in the case of GS, were higher in cattle than in buffaloes. The specific activity of AS was significantly higher in cattle than in buffaloes. In cattle the specific activity of AS was considerably higher than that of GS. Of the electrolytes tested, $MnCl_2$ (10 mmol/l) and $CoCl_2$ (1 mmol/l) increased GS activity by 16 and 117%, respectively, while NaCl (50 mmol/l), KCl (50 mmol/l), $CaCl_2$ (10 mmol/l), NH_4Cl (50 mmol/l), and $MnCl_2$ (10 mmol/l) increased AS activity by 20, 9, 25, 29 and 39%, respectively, in both species as compared to the control conditions. Other chemicals such as EDTA, ammonium molybdate, sodium arsenate, sodium azide and PCMB at 1 mmol/l concentration each reduced the activity of GS and AS by 42 and 41, 51 and 58, 39 and 39, 91 and 50, 51 and 44%, respectively.

Keywords: Glutamine synthetase, asparagine synthetase, rumen bacteria, ruminants, buffalo, cattle.

In many organisms like bacteria (Ravel et al., 1962; Bender et al., 1977; Siedel and Shelton, 1979) and algae (Tuli and Thomas, 1980), ATP-dependent glutamine synthetase (GS) and asparagine synthetase (AS) are important enzymes for the fixation of ammonia into amide of glutamine and asparagine, respectively. These amide groups may further be transferred to suitable carbon skeletons for the synthesis of other amino acids. The presence of these enzymes in mixed microorganisms (Chalupa et al., 1970; Erfle et al., 1977) and pure culture of bacteria (Burchall et al., 1964; Smith et al., 1980) from the rumen has also been detected but not fully characterized. This study deals with the characterization of GS and AS from the mixed rumen bacterial flora of buffaloes and cattle.

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Materials and methods

The animals, their feeding schedule and the procedure of collecting rumen fluid (RF) were the same as described in the accompanying paper (Saini and Singh, 1988). The crude enzyme extract was prepared (Baldwin and Palmquist, 1965) from 1 l of cold RF and stored at 0 °C in small vials (0.5 ml). The enzyme preparation, termed mixed rumen bacterial extract (MRBE), was stable for several days when stored at 0 °C.

The GS (Chalupa et al., 1970; EC 6.3.1.2) and AS (Burchall et al., 1964; EC 6.3.1.1) activity in MRBE was measured by determining, respectively, the formation of Y-glutamyl hydroxamate and β-aspartyl hydroxamate. The reaction mixtures (2.25 ml final volume), consisting of sodium glutamate, 120 μmol; potassium arsenate, 80 μmol; manganese chloride, 12 μmol; hydroxylamine hydrochloride (neutralized with KOH), 240 μmol; imidazole (pH 7.5) 80 μmol; sodium ATP, 10 μmol; and MRBE, 0.1 ml (all the substrates were prepared in imidazole buffer of pH 7.5) for GS, and L-aspartate, 20 μmol; tris (hydroxymethyl) aminomethane-hydrochloride (Tris-HCl), 100 μmol; manganese chloride, 12 μmol; sodium ATP, 10 μmol; hydroxylamine hydrochloride (neutralized with KOH), 800 μmol; and MRBE, 1.0 ml (all the substrates were prepared in Tris-HCl buffer of pH 7.2) for AS, were incubated for 60 min at 37 °C. 0.75 ml solution containing equal volumes of 24% (w/v) trichloroacetic acid, 6 N HCl and 10% (w/v) $FeCl_3 \cdot 6\ H_2O$ in 0.02 N HCl, was added to stop the reaction. The optical density was measured (after centrifugation at 1000 *g* for 10 min) at 540 nm in a Spectronic-20 spectrophotometer (Bausch and Lomb). Appropriate controls were always included in the assay to estimate the non-enzymatic activity and subtracted from the experimental values. Quantitation of Y-glutamyl hydroxamate and β-aspartyl hydroxamate was done from their standard curves prepared in the range of 1.0 to 10.0 μmol. The specific activities of GS and AS were expressed as nmol of product formed $h^{-1}\,mg^{-1}$ protein. The protein content of MRBE was determined by the method of Lowry et al. (1951). The K_m values were determined by conventional methods (Dixon and Webb, 1964).

Results

The GS from both buffaloes and cattle has pH optima of 7.6. In MRBE from buffaloes the activity at pH 7.2, 6.4, 8.2 and 8.6 was reduced by 25.0, 52.5, 22.5 and 46.2%, respectively. The corresponding reductions in cattle were 25.6, 50.6, 13.7 and 52.7%. The optimal concentration (mmol/l) of sodium glutamate and sodium ATP was 150 and 4, respectively, in the preparations from both buffaloes and cattle (Table I). Increasing concentrations of both

substrates beyond their optimal levels inhibited the enzyme activity in both ruminant species. The K_m values in buffaloes and cattle for these substrates are given in Table III.

The AS has pH optima of 7.2 and 7.0 in MRBE from buffaloes and cattle, respectively. In MRBE from buffaloes the activity at pH 6.8, 6.0, 8.0 and 8.4 was reduced by 4.5, 48.9, 26.1 and 54.5%, respectively. The corresponding reductions in cattle were 2.0, 55.9, 21.6 and 51.0%. The optimal concentration (mmol/l) of potassium aspartate and sodium ATP for the enzyme activity was 50 and 6, respectively, in MRBE from both ruminant species (Table II). Increasing concentrations of both these substrates beyond their optimal levels

Table I

Effect of different concentrations of substrates on the activity of GS (nmol Y-glutamyl hydroxamate produced h^{-1} mg^{-1} protein) in the extracts of mixed rumen bacteria from buffaloes and cattle (n = 2 each)

Sodium glutamate (mmol/l)	Buffaloes	Cattle	Sodium ATP (mmol/l)	Buffaloes	Cattle
12.5	95.6	103.5	0.5	78.7	81.0
25.0	151.9	153.0	1.0	135.0	148.5
50.0	174.4	202.5	2.0	213.7	265.5
100.0	208.1	274.5	4.0	270.0	324.0
150.0	264.3	315.0	6.0	219.3	247.5
175.0	236.2	225.0	8.0	180.0	216.0
200.0	202.5	198.0	12.0	112.5	135.0

In Tables I and II, except for the substrates whose concentration is varied, the concentration of all other substrates was the same as given under Materials and methods

Table II

Effect of different concentrations of substrates on the activity of AS (nmol β-aspartyl hydroxamate produced h^{-1} mg^{-1} protein) in the extracts of rumen bacteria from buffaloes and cattle (n = 2 each)

Potassium L-aspartate (mmol/l)	Buffaloes	Cattle	Sodium ATP (mmol/l)	Buffaloes	Cattle
6.25	112.5	360.0	0.5	168.7	629.9
12.5	213.7	755.9	1.0	236.2	899.9
25.0	281.2	989.9	2.0	281.2	1079.9
50.0	326.2	1169.9	4.0	315.0	1169.9
75.0	202.5	539.9	6.0	337.5	1259.9
100.0	157.5	323.9	8.0	292.5	989.9
150.0	101.2	144.0	12.0	225.0	809.9
			16.0	146.2	548.9

Table III

K_m and V_{max} values (determined by Lineweaver-Burk plots) of GS and AS in the extracts of mixed rumen bacteria from buffaloes and cattle (n = 2 each; mean ± SD)

Enzyme	Substrate	K_m (mmol/l)		V_{max} (nmol product formed h^{-1} mg^{-1} protein)	
		Buffaloes	Cattle	Buffaloes	Cattle
GS	Sodium glutamate	54.05 ± 3.01	51.28 ± 3.61	270.37 ± 10.18	316.67 ± 15.11
	Sodium ATP	1.96 ± 0.08	2.50 ± 0.12	280.00 ± 15.04	330.32 ± 18.21
AS	Potassium L-aspartate	10.42 ± 1.01	11.36 ± 1.18	340.16 ± 20.21	1180.57 ± 59.81
	Sodium ATP	0.94 ± 0.09	1.14 ± 0.07	345.12 ± 26.02	1278.60 ± 65.13

Table IV

Effect of different electrolytes on the activity of GS and AS in the extracts of mixed rumen bacteria from buffaloes and cattle (n = 2 each)

Electrolyte (mmol/l)	GS		AS		Electrolyte (mmol/l)	GS		AS	
	Buffalo	Cattle	Buffalo	Cattle		Buffalo	Cattle	Buffalo	Cattle
$NaCl_2$, 50	70	67	119	122	$CoCl_2$, 1	218	216	75	81
KCl, 50	68	71	109	109	$NiCl_2$, 1	66	64	63	69
$CaCl_2$, 10	66	65	125	125	$ZnSO_4$, 1	80	74	100	100
NH_4Cl, 50	75	73	131	128	$CuSO_4$, 1	57	52	71	69
$MnCl_2$, 10	114	118	138	140	$CdSO_4$, 1	86	82	102	104
K_2HPO_4, 50	36	34	23	19	$HgCl_2$, 1	64	57	15	12

The figures are adjusted by taking the value of control (without electrolyte) as 100

Table V

Effect of some chemicals on the activity of GS and AS in the extracts of mixed rumen bacteria from buffaloes and cattle (n = 2 each)

Chemical (mmol/l)	GS		AS		Chemical (mmol/l)	GS		AS	
	Buffalo	Cattle	Buffalo	Cattle		Buffalo	Cattle	Buffalo	Cattle
EDTA, 1	61	55	60	58	Sodium arsenate, 1	64	58	59	62
Ammonium molybdate, 1	48	50	42	42	Sodium azide, 1	9	9	50	50
Sodium citrate, 1	105	105	108	111	PCMB, 1	52	45	56	56

The figures are adjusted by taking the value of control (without chemical) as 100

decreased the reaction velocity. K_m values of the enzyme for these substrates are given in Table III.

The specific activity of GS (when the concentrations of the substrates were the same as described in the Materials and methods section) was 4.4

and 5.2 in MRBE from buffaloes and cattle, respectively. The corresponding values of AS were 5.4 and 19.5. These data and the results of Table II show that the specific activity of AS was considerably higher in MRBE from cattle than in that from buffaloes. It is also seen that the specific activity of AS was much higher than that of GS in preparations from cattle.

$MnCl_2$ (10 mmol/l) and $CoCl_2$ (1 mmol/l) increased GS activity by 14–18 ($P < 0.1$) and 116–118% ($P < 0.01$), respectively, while NaCl (50 mmol/l), KCl (50 mmol/l), $CaCl_2$ (10 mmol/l), NH_4Cl (50 mmol/l), $MnCl_2$ (10 mmol/l) increased AS activity by 19–22 ($P < 0.1$), 9, 25 ($P < 0.1$), 28–31 ($P < 0.1$) and 38–40% ($P < 0.05$), respectively, over the control values. $ZnSO_4$ (1 mmol/l), $CdSO_4$ (1 mmol/l) in the case of AS and $LiSO_4$ (1 mmol/l) in the case of both GS and AS were without effect. Other salts had an inhibitory effect (Table IV). The effect of various chemicals on the activity of GS and AS were also tested (Table V). EDTA, ammonium molybdate, sodium arsenate, sodium azide and PCMB at 1 mmol/l concentration each inhibited the activity of GS and AS by 39–45 and 40–42, 50–52 and 58, 36–42 and 38–41, 91 and 50, 48–55 and 44%, respectively, in both ruminant species. Sodium citrate (1 mmol/l) slightly activated both enzymes.

Discussion

The presence of GS, AS and pyridine nucleotide linked glutamate synthase (GOGAT) in mixed (Chalupa et al., 1970; Erfle et al., 1977) and pure cultures of rumen bacteria (Burchall et al., 1964; Griffith and Carlsson, 1974; Smith et al., 1980) suggested that these enzymes may provide an efficient means of glutamate synthesis through the glutamate or asparagine/glutamate pathway especially at low ammonia concentration in the rumen. Glutamate in turn can play an important role as the donor of amino groups in the synthesis of other amino acids (Umbarger and Davis, 1962). The specific activities and K_m values of GS and AS (Table III) and of glutamate dehydrogenase (Saini and Singh, 1987), glutamic-oxaloacetic transaminase and glutamic-pyruvic transaminase (Saini and Singh, 1988) reported earlier in the MRBE from buffaloes and cattle indicated that energy-dependent fixation of ammonia by rumen bacteria through GS and AS may be of lesser importance in these two ruminant species. Similar were the views of Chalupa et al. (1970) and Wallace (1979). It is interesting to note that the specific activity of AS was significantly higher in MRBE from cattle than in that from buffaloes, and the specific activity of AS was higher than that of GS in preparations from cattle.

The results on the effect of various electrolytes and chemicals (Tables IV and V) on the activity of GS and AS indicate that their activities in the rumen can be manipulated.

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METALLOTHIONEIN CONTENTS IN SELECTED TISSUES OF CATTLE FROM COPPER-DEFICIENT AREAS IN NORTH-EAST POLAND*

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Investigations were carried out on 90 cows and 30 young bulls, of the Black and White breed, allotted into four groups, in three Cu-deficient regions (A, B, C) of Poland. The first task was to determine Cu, Zn and Cd levels in the blood. Furthermore, five animals in each group were slaughtered to determine metallothionein as well as Cu, Zn and Cd in the liver, kidney, epithelial tissues of the rumen, reticulum, omasum, abomasum, duodenum, jejunum, ileum and colon. These metals were also determined in the soil and herbage.

The contents of metallothionein in the tissues examined ranged from 0.07 to 0.72 mg/g. The highest concentrations (0.19–0.52 mg/g) were measured in the kidney, liver, epithelial tissues of the rumen, reticulum, omasum and abomasum of bulls from regions where the highest Cd levels were found (C), and in the duodenum and jejunum (0.48–0.59 mg/g) of cows kept in areas with the highest Zn contents (A).

Keywords: Metallothionein, cattle, copper, zinc, cadmium.

Metallothionein is a protein of low molecular weight (about 10,000) and high sulphur content, binding also Cu, Zn, Cd, Hg and Fe (Kägi and Vallee, 1960; Kägi and Vallee, 1961). The total quantity of metals bound to the protein amounts to 6–8 gram atoms per 6000 g metallothionein. However, the contents of metals often vary and depend on the size of minerals delivered as well as on the organ (Piotrowski and Mogilnicka, 1976). Experimental data showed that the synthesis of metallothionein could be induced in the tissues by metals such as Cu, Zn, Cd (Piscator, 1964; Kägi and Nordberg, 1979), Hg, Bi, Co (Piotrowski and Mogilnicka, 1976) and Ag (Mogilnicka et al., 1976). If healthy animals are supplemented with Cu or Zn, metallothionein content will increase and, subsequently, these metals will accumulate in the liver, kidney, intestines and other tissues (Mercer et al., 1981; McCormick et al., 1981; Menard et al., 1981). However, deficient doses of Cu or Zn decrease the level of metallothionein so that it is sometimes undetectable (Cousins, 1985; Richards and Cousins, 1976).

The role of metallothionein in the liver, intestines and kidney is not quite clear. The binding of trace elements may be important for the homeostasis

* *Editor's note:* Since the cadmium concentrations apply to a circumscribed geographic area, the conclusions drawn from them cannot be generalized.

of Cu and Zn in the body as well as for protection against toxic levels of minerals (Pattison, 1984; Weiner and Cousins, 1980). Cousins (1985) suggests a scheme of Cu and Zn economy in which metallothionein has the central position. It seems, however, that other functions of the protein are also possible. Prolonged uptake of Cu and Zn in large quantities renders animals resistant and improves their defence mechanisms through changes in metallothionein levels (Powanda, 1981). Moreover, the hepatic metallothionein can bind gold strong enough (Sharma and McQueen, 1982); thus, the success of anti-inflammatory treatment with its salts may depend on the content of this protein. Platinum is also able to bind metallothionein (Żelazowski et al., 1984), thus inhibiting the therapeutic efficiency of antineoplastic drugs containing this metal (Bakka et al., 1981).

Information on the physiological levels of metallothionein in the tissues has been incomplete and contradictory so far, while for cattle there is no such information whatsoever. Therefore, the aim of this work was to present some data on the protein contained in selected tissues of cattle from Cu-deficient regions of North-east Poland.

Materials and methods

The experiment was performed on 90 cows aged 3–6 years, with a milk yield about 3000 l per year, and on 30 bulls aged 18 months. All the animals were of the Black and White breed. Taking into account regional conditions, the animals were allotted into four groups (I, II, IIIa, IIIb). There were 30 cows in region A (Group I) and 30 cows in region B (Group II), while in region C there were 30 bulls (Group IIIa) and 30 cows (Group IIIb). During the grazing season the animals were kept on pasture, while in winter their diet consisted of grass silage and hay.

Blood samples were taken twice a year from all the animals to determine Cu, Zn and Cd levels in the serum. Moreover, in each group five animals were slaughtered to take samples from the liver, kidney, epithelial tissues of the rumen, reticulum, omasum, abomasum, duodenum, jejunum, ileum and colon for determination of metallothionein, Cu, Zn and Cd contents. The metals were also determined in samples of soil and herbage taken from particular regions. Copper, zinc and cadmium were determined by atom-absorption spectrophotometry while for metallothionein a radioisotope method using Hg^{203} (Żelazowski and Piotrowski, 1977) was used. The results were evaluated statistically by Student's t test.

Table I

Metallothionein, Cu, Zn and Cd contents in selected tissues

Groups	Parameter	Organs or tissues										
		1	2	3	4	5	6	7	8	9	10	11
I	MT	0.35	0.38	0.10	0.12	0.09	0.18	0.48*	0.59*	0.32	0.15	—
	Cu	58.9	479	44.5	39.8	30.8	33.6	39.7	37.5	37.6	36.2	8.88
	Zn	269	291	286	254	236	244	258	233	209	210	14.0
	Cd	0.84	0.55	0.52	0.50	0.44	0.42	0.46	0.43	0.36	0.49	0.008
II	MT	0.31	0.47	0.07	0.10	0.10	0.15	0.33	0.21	0.16	0.35	—
	Cu	95.8	535	43.2	40.7	42.6	41.7	44.5	46.3	42.1	40.2	8.05
	Zn	227	388	268	294	267	229	259	246	207	216	11.9
	Cd	0.72	0.43	0.43	0.47	0.34	0.33	0.52	0.37	0.47	0.43	0.008
IIIa	MT	0.52*	0.72*	0.19*	0.16*	0.25*	0.19	0.22	0.40	0.43	0.28	—
	Cu	70.6	779**	47.7	43.6	44.3	46.9	48.9	47.2	37.6	34.7	7.90
	Zn	383	434*	293	267	288	168	188	267	188	196	12.8
	Cd	1.11*	0.78*	0.58	0.48	0.44	0.62	0.83	0.47	0.38	0.52	0.008
IIIb	MT	0.46	0.50	0.12	0.15	0.17	0.18	0.39	0.27	0.23	0.20	—
	Cu	74.8	611	45.6	42.5	43.2	39.7	48.3	46.6	43.3	32.7	7.65
	Zn	298	399	332	287	264	196	207	255	213	216	12.6
	Cd	0.71	0.46	0.41	0.42	0.37	0.30	0.42	0.35	0.42	0.44	0.008

Explanations: Values of metallothionein are given in [mg/g]. Values of Cu, Zn and Cd in tissues are given in [μmol/kg]., while in serum in [μmol/l]. MT: metallothionein, 1: renal cortex, 2: liver, 3: epithelium of rumen, 4: epithelium of reticulum, 5: epithelium of omasum, 6: epithelium of abomasum, 7: epithelium of duodenum, 8: epithelium of jejunum, 9: epithelium of ileum, 10: epithelium of colon, 11: serum.

* Statistically significant difference; ** statistically highly significant difference

Results

The contents of metallothionein, Cu, Zn and Cd in the renal cortex, liver, epithelial tissues of the rumen, reticulum, omasum, abomasum, duodenum, jejunum, ileum, colon and serum are shown in Table I. The highest contents of metallothionein were found in the liver, kidney, epithelial tissues

Table II

Cu, Zn and Cd contents in soil and herbage

Metals	Regions					
	A		B		C	
	Soil	Herbage	Soil	Herbage	Soil	Herbage
Cu	3.00	2.60	4.90	3.30	3.60	3.00
Zn	67.0*	33.6	28.0	22.9	43.0	27.8
Cd	0.09	0.06	0.08	0.02	0.10	0.16*

Explanations: the values found in soil are given in mg/kg, while those measured in herbage in mg/kg dry matter; * statistically significant difference

of the rumen, reticulum, omasum and abomasum of bulls from Group IIIa. For the kidney, liver, rumen, omasum, and abomasum the differences were statistically significant ($P < 0.05$), in relation to the values found in similar tissues of animals from Groups I, II, or IIIb. The highest contents of metallothionein were found in the duodenum and jejunum of cows from Group I. The difference was statistically significant ($P < 0.05$), in comparison with the values obtained for Groups II, IIIa and IIIb.

The highest contents of Cd were found in the kidney and liver of bulls from Group IIIa. These values were significantly ($P < 0.05$) higher than those obtained for Groups I, II, and IIIb. Also, the Cu and Zn contents in the liver of animals from Group IIIa were highly significantly ($P < 0.01$) and significantly ($P < 0.05$) higher than those found in Groups I, II and IIIb.

The data on Cu and Cd concentrations in soil and herbage are contained in Table II. The highest Zn concentrations were found in region A in both soil and herbage ($P < 0.05$), while the highest Cd concentration was measured in the herbage of region C.

Discussion

The highest metallothionein content in the kidney, liver, rumen, reticulum, omasum and abomasum was found in bulls (Groups IIIa) from region C. The highest concentration of cadmium was also found in the kidneys and livers of these animals (Table I). It seems that the results are due to the

inductive influence of this metal, which was found in the highest quantities in the soil and herbage of region C (Table II). This observation is supported by data of the literature published so far on increased synthesis of metallothionein as a result of the long-term influence of Cd (Kägi and Nordberg, 1979). Therefore, enhanced induction of metallothionein by Cd seems to be interesting, however, in relation to the rumen, reticulum, omasum and abomasum only. In organs with very intensive absorption, i.e. in the duodenum and jejunum, also high metallothionein contents were found, but only in cows from Group I, region A, where the highest Zn concentrations were found in both soil and herbage. Some researchers reported that large amounts of zinc should be given to animals for a long time to induce increased levels of hepatic metallothionein (Chen et al., 1977; Oh et al., 1978). It is easier to induce an increased level of metallothionein with zinc in the epithelial tissues of the duodenum and jejunum than in other organs of cattle. As far as induction of metallothionein by copper is concerned, the problem is quite different. In the present survey low levels of Cu were found in the soil and herbage of all regions examined. However, the lowest levels of this element as well as of metallothionein (0.38 mg/g) were recorded in the livers of cows from group I. This fact may point to a joint action of hepatic copper and metallothionein contained in this organ (Bremner and Young, 1976; Cousins, 1985). The present work should be considered a preliminary one. Further publications will furnish more data on the biosynthesis of metallothionein and on how it is influenced by some trace elements.

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NARASIN POISONING IN TURKEYS

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Mass disease and a considerable mortality rate (over 32%) are reported from a turkey flock of 3000 birds aged 11 weeks. As a cause, narasin poisoning has been incriminated.

The most characteristic clinical signs were locomotor disorders. These were accompanied by severe dyspnoea and moderate diarrhoea. At necropsy, in the mass muscles there was muscle fibre damage, in most cases visible with the unaided eye. Histologically, the lesions proved to be Zenker's degeneration and necrosis. In addition catarrhal enteritis, renal degeneration and, in several cases, pulmonary congestion were observed. Nearly one-third of the flock died within one week after the clinical signs first appeared. The highest mortality occurred among the toms.

The ration consumed by the turkeys had contained 42.8 mg/kg (40–50 mg/kg) narasin, slightly "contaminated" with monensin.

In feeding trials conducted with batches of the original ration containing somewhat less ionophore antibiotics (about 28 mg/kg), the clinical signs and gross lesions characteristic of narasin toxicity could be elicited in turkeys of similar age (10 weeks) as in the field cases; however, it took longer for the clinical signs to appear than in the affected flock.

The present results support the observations of British authors (Davis, 1983; Simpson, 1984) that turkeys are highly sensitive to narasin, and that rations containing a small fraction of the therapeutic narasin dose established for broiler chickens might cause considerable losses among turkeys.

Keywords: Turkey, narasin, toxicity.

Coccidiosis not infrequently causes severe losses among young turkeys, justifying the preventive use of anticoccidial drugs. For this purpose preparations containing halofuginone, clopidol, amprolium and monensin are commercially available in Hungary. Thus, out of the so-called ionophore anticoccidials only monensin has been licensed for use. The main reason why other ionophores have not been licensed for use in turkeys is that they are rather toxic to that species.

On the occasion of field cases among turkeys, several authors have reported incidences of ionophore toxicosis caused by monensin (Braunius, 1986; Halvorson et al., 1982; Sályi, 1985; Stuart, 1978), salinomycin (Halvorson et al., 1982; Stuart, 1983) and narasin (Davis, 1983; Simpson, 1984). Other researchers (Potter et al., 1986; Weisman et al., 1980) studied such cases under experimental conditions.

There are very few data in the literature on toxicosis caused by narasin

(Monteban, Eli Lilly Co.) in farm animals. We have diagnosed narasin toxicosis in turkeys on six occasions. In this paper a typical case of mass narasin poisoning and its experimental reproduction are presented.

Materials and methods

Feed analysis. The ionophore antibiotic content of the feed was determined by a method described by Golab et al. (1973). Narasin was identified, and differentiated from other ionophore antibiotics, by the thin-layer chromatographic procedure reported by Owles (1984), modified as follows: to ensure a reliable separation of narasin and salinomycin, the extract applied onto the chromatographic plate was run first in diethyl ether, then in a 98 : 2 mixture of ethyl acetate and water.

Histopathological examination. The liver, kidneys, heart, skeletal muscle portions, pancreas, brain, spinal cord segments (cervical, lumbar), brachial plexus, and sciatic nerves (n. ischiadicus) of numerous growing turkeys that had died, or had been killed, during the field case of poisoning and the feeding trial, were fixed in 10% neutral formalin. Frozen and paraffin-embedded sections were prepared and stained with P. Mayer's acidic haemalaun and eosin. The lipids were detected by the Fettrot staining.

Clinico-chemical tests. The activities of various enzymes of tissue origin, viz. creatine kinase (CK; EC 2.7.3.2), aldolase (ALD; EC 4.1.2.13), lactate dehydrogenase (LDH; EC 1.1.1.27) and alpha-hydroxybutyrate dehydrogenase (HBDH; EC 1.1.1.27) were measured in the serum of the experimental turkeys by clinico-chemical methods. The determinations were performed by using Boehringer test collections and an Eppendorf type ACP 5040 instrument, as recommended by the International Society of Animal Clinical Biochemistry.

Results

Case report

On the 2nd day after the feed had been changed, in a turkey flock of more than 3000 birds of the BUT breed, aged 11 weeks, part of the birds became listless, were reluctant to move and most of them became prostrate with legs curled up. The number of affected turkeys rose rapidly and by the evening 77 birds had died. The turkeys had moderate diarrhoea and excreted light yellowish-white faeces.

By the morning of the 3rd day about 80% of the birds were "sitting" and most of them were unable to lift their heads from the floor. Other birds were standing unsteadily, tried to get up reclining on their wings, or fluttered

them. It was striking that the turkeys had lost their voice. The rate of mortality kept increasing, arousing the suspicion of feed toxicosis. Therefore, in the afternoon the incriminated feed was withdrawn and replaced by a new ration.

On-the-spot investigations could not exclude a bacterial aetiology. Therefore, from the third day the turkeys received Neo-Sulfotrim® (active substance: sulfachloropyridazine sodium, sulfamethoxazole and trimethoprim) treatment for three days, followed by Chlorocid (chloramphenicol) therapy of the same duration. The antibiotics were administered via the drinking water.

No appreciable improvement occurred after withdrawal of the suspect feed. Nor did antibiotherapy cause a change for the better. The clinical signs subsided only gradually, the number of deaths even kept increasing for a while before it began to decrease. The daily incidence of deaths is shown in Fig. 1.

It was interesting to note that while originally the proportion of toms had been about 40% in the flock, after the disease it was only 3–4%, indicating that mainly male birds had died.

On day 3 and 6 after the birds started to receive the suspect ration, 20 dead and 3 severely affected turkeys were submitted to our institute for further examination. *Gross pathological examination* at necropsy revealed enteritis, renal degeneration, and pale striations in certain thigh muscles. In some cases the myocardium was paler than usual. In one bird the cavities of the

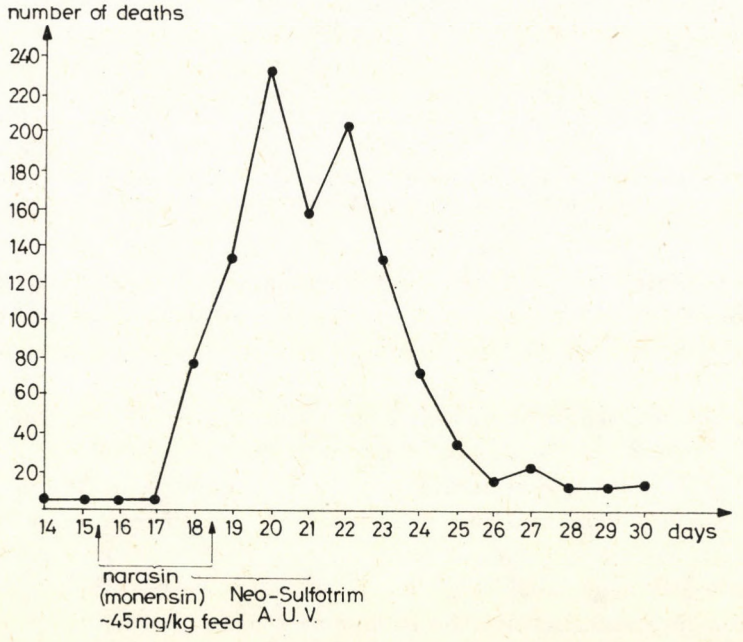


Fig. 1. Duration of feeding narasin-containing and medicated feed and the daily number of deaths

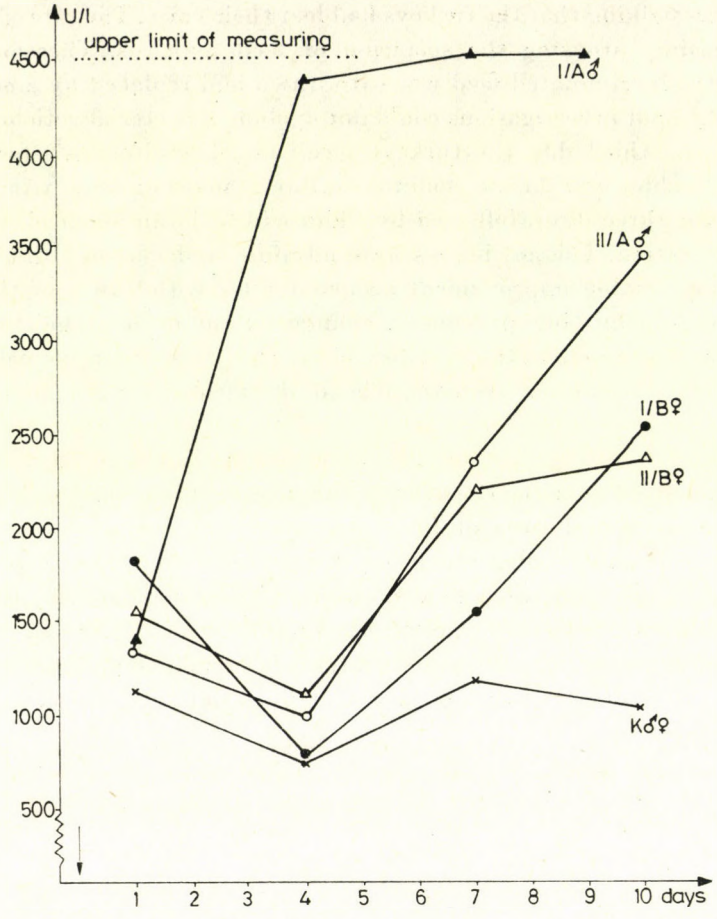


Fig. 2. Serum creatine kinase (CK) activity in the experimental group of turkeys (arrow: start of feeding narasin-containing (28 mg/kg) feed)

heart were dilated, its wall became thinner, and other pathological changes suggestive of cardiac dilatation (e.g. presence of straw-coloured, easily coagulating contents in the pericardium and abdominal cavity) were also observed.

Histopathologically, there was Zenker's degeneration and necrosis of the myofibrils (Fig. 3) in the various thigh muscles, but always in m. pectineus. This extended to a varying number of myofibril bundles. In the myocardium here and there oedematous infiltration and muscle fibre atrophy were seen.

Bacteriological examination of the organs (liver, heart) of the dead birds was consistently negative.

The sample taken from the feed shortly after the disease emerged and submitted to our institute for *toxicological examination* did not contain de-

tectable amounts of T-2 fusariotoxin and furazolidone. The NaCl content of the sample was as prescribed (0.26%). On the basis of the total germ and mould counts the microbiological status of the feed sample was not objectionable. By thin-layer chromatography the sample was found to contain 75 mg/kg dimetridazole and 42.8 mg/kg ionophore anticoccidials (mainly narasin, and, as "contaminant", monensin).

Subsequently new samples were taken from the incriminated feed stock and the toxicological examination was repeated. The ionophore anticoccidial content of 10 different sacks ranged between 25.2 and 37.5 mg/kg (extreme values), most (about 80%) of which was narasin and the remaining 20% was monensin. It should be mentioned that, as it was found out later, three other farms had also received feed from that batch. At one of those three farms a disease of similar type and severity occurred. At another, where the feed had been fed only for one day, no clinical signs occurred. At the third farm the feed had been fed for two days. Here locomotor disturbances did occur; however, the losses were moderate.

In the case presented above, the case history, the course of the disease, the clinical signs, the gross and histopathological changes and the presence of narasin in the feed sample all pointed in the direction of narasin poisoning. As few data had been available on the sensitivity of turkeys to narasin, and

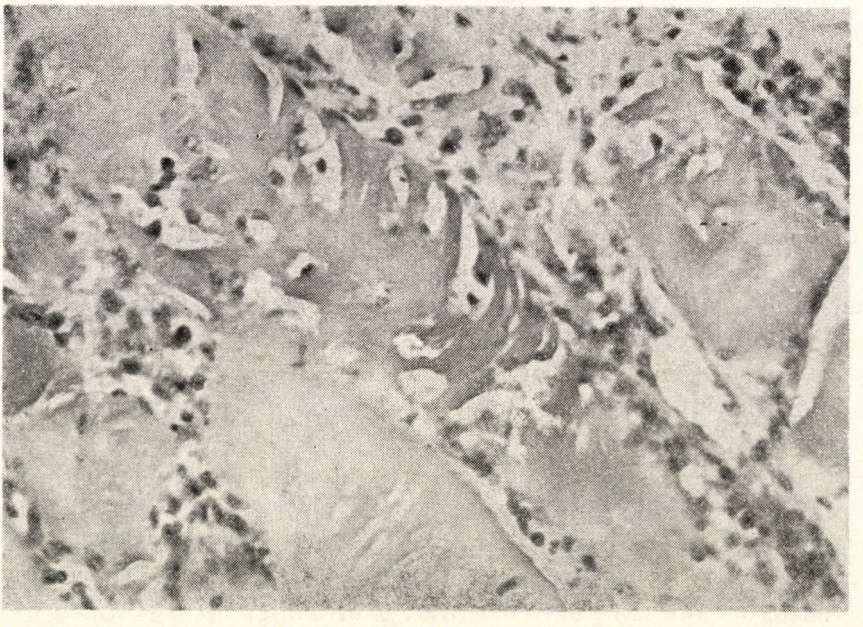


Fig. 3. Zenker's necrosis of myofibrils, with infiltration by inflammatory cells in the disrupting muscle fibre and proliferation of angiofibroplast tissue in the endomysium. Haematoxylin and eosin, × 160

since the narasin concentration of the feeds was moderate (30–40 mg/kg), we attempted to reproduce the disease under experimental conditions by feeding the suspect ration.

Feeding trial

Twenty turkeys of the BUT breed, aged 10 weeks, were used. Birds of Group I (8 turkeys) received the "suspect" diet collected on the spot of the field cases. Turkeys of Group II (8 birds) received the same feed plus, as in the field case, Neo-Sulfotrim dissolved in the drinking water at a dose rate corresponding to 10 mg sulfonamide active substance/kg body mass. In this experimental set-up we wanted to determine whether or not the administration of Neo-Sulfotrim aggravated the clinical signs of poisoning.

Both groups contained the same number of male (Sub-groups I/A and II/A) and female (Sub-groups I/B and II/B) birds. The four turkeys of Group C served as control: they received a ration from another production batch.

On the average of several measurements the feed of Groups I and II contained 28 mg/kg ionophore coccidiostats, with a narasin/monensin ratio similar to that found in earlier investigations.

The first clinical signs appeared on day 4 of the experiment. Toms of Sub-group I/A were mainly "sitting". When attempting to get up, they invariably dropped back after a short time. The toms of both groups had lost their voice.

On day 5 a turkey of Sub-group I/A moved unsteadily, while another hung its wings and, when slightly overbalanced, reclined on both wings to preserve the balance. By day 6 all turkeys of that group developed dyspnoea, breathed with beaks agape, and their comb as well as skin of neck had become cyanotic. Most birds were sitting; when raised, they could remain in standing position only for a short time. When overbalanced, they reclined on their side. Some birds attempted to walk with legs sinking under them and tried to balance themselves with their wings. Although in a less severe form, these signs were observed in Sub-group II/A, too. At the same time, disregarding dyspnoeic signs in some of them, the hens (Sub-groups I/B and II/B) remained asymptomatic throughout the two-week observation period.

By the morning of day 7 two turkeys of Sub-group I/A had become prostrate, were unable to stand up and lay with legs curled up or outstretched. Two other birds hung their wings (Fig. 4) and moved very unsteadily. At that time all four turkeys of Sub-group II/A were hanging their wings. By the afternoon the two severely affected turkeys of Sub-group I/A had died.

On day 9 the two surviving turkeys of Sub-group I/A, which by that time had developed locomotor disturbances, were killed for gross and histopathological examination.

In Sub-group II/A the locomotor disturbances persisted throughout the 14-day experimental period, though they slightly subsided in the last days of the experiment.

During the experiment the turkeys fed on the narasin- (and monensin-) containing ration lost some weight despite the fact that their feed intake hardly differed from that of the control birds which grew normally.

In the pectoral, thigh adductor and wing muscles of turkeys that had died, or had been killed, during the experiment degenerated areas of varying size were visible even with the unaided eye. Histologically, these lesions corresponded to various stages of Zenker's necrosis of the myofibrils, depending on the time of death.

Alteration in the enzyme activities (CK, LDH) of the blood serum and the inter-group differences existing in enzyme activities were in good agreement with the severity of the clinical symptoms. Creatine kinase (CK) activity is shown in Fig. 2. Initially CK activity had been slightly higher than usual. This must have been due to the slight muscular damage which had occurred during transportation of the turkeys immediately before the experiment. (It should be noted that the serum CK activity of Sub-group I/A birds might have been considerably higher than the value indicated in the figure, for 4500 U/l is the upper limit of concentration measurable by the instrument applied; we did not repeat the determination with diluted serum.)



Fig. 4. Turkey hanging its wings on day 5 of the experiment

The results show that muscular damage develops, though later and in a less severe form, also in the turkey-hens. ALD and HBDH activities are difficult to interpret because of the small number of samples and the great individual variation.

Discussion

The clinical signs and pathomorphological changes found in the field case were successfully reproduced under experimental conditions by feeding the "suspect" ration. The clinical signs and pathological changes resembled those reported for subacute ionophore anticoccidial poisoning among turkeys (Halvorson et al., 1982; Sályi, 1985; Stuart, 1978, 1983). The results of the feeding trial prove that the given feed was toxic, and confirm the suspicion of narasin poisoning.

This conclusion is supported by the observations of Davis (1983) who reported that feeds of 70 mg/kg narasin content were highly toxic to turkeys of nearly identical age as those in the present case. The clinical signs described by him were consistent with those found by us in both the field case and the feeding trial. As evidenced by a field case that has occurred since our experiment was finished, in turkeys rations containing half the narasin concentration recommended for broiler chickens as preventive dose (around 35 mg/kg) can produce the above disease entity. Based on this observation we can rule out the hypothesis that monensin traces present in the feed significantly enhanced the toxicity of the moderate narasin content of the ration, and this gave rise to toxicity.

Toms were more severely affected, and more deaths occurred among them than among the hens, in both the field case and the feeding trial. This tallies with the observations of Davis (1983). The considerable sex-dependence existing in the turkeys' sensitivity to narasin may apply to other ionophores, and other fowl species, as well. Namely, during a recently diagnosed incidence of monensin poisoning in a breeding flock of chickens almost all the cocks died, whereas in the laying hens the poisoning was characterized by severe locomotor disturbances and a significant drop in egg production, both of which were, however, transient.

In the turkey flock studied, the daily incidence of deaths, i.e. the fact that after withdrawing the narasin-containing feed the mortality rate kept increasing and deaths continued to occur for almost a week, is consistent with earlier observations made by us (Sályi, 1985) and those reported by Stuart (1978, 1983) in connection with monensin and salinomycin poisoning in turkeys.

We cannot give a satisfactory explanation for the observation that, contrary to our expectations based on the results of other authors (Braunius, 1986; Simpson, 1984), the disease was less severe in the medicated group.

Simpson (1984) reported that by giving sulfaquinoxaline to turkeys fed low concentrations (about 10 mg/kg) of narasin, the toxic signs typical of ionophore poisoning could be elicited. Recently Laczay et al. (1987) have reported that *only certain* sulfonamides are able to potentiate the toxicity of ionophores. As shown by feed intake data, the medicated turkeys consumed the same amount of feed as the non-medicated ones.

The absence of the expected toxicity-potentiating effect may partly be explained by the fact that in our experiment the sulfonamides were dissolved in the amount of drinking water intended for the whole day, contrary to the general practice that drugs are usually dissolved in the drinking water volume which the birds consume in one or two hours.

We were unable to reveal the fault of mixing technology as a result of which narasin had been mixed in the turkeys' feed.

According to other authors (Halvorson, 1982; Stuart, 1983) and also in our experience (Sályi, 1985), incidences of poisoning caused by the different ionophore antibiotics cannot be differentiated on the basis of the clinical signs and gross and histopathological changes seen at necropsy.

Acknowledgements

The authors thank Dr. Ferenc Rátz for his help in the histopathological examination.

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SCANNING ELECTRON MICROSCOPIC EXAMINATION OF CARTILAGE IN CHICKEN EMBRYOS TREATED WITH THE INSECTICIDE WOFATOX 50 EC

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Fertile hen's eggs were treated with the insecticides Wofatox 50 EC (50% methyl-parathion) or Parathion 20 WP (latter as positive control material). The eggs were exposed by immersion technique before incubation or on the 4th day of the hatching period. Cartilage samples were taken out of the vertebrae (C_{1-7}) and phalanges (P_{1-11}) of control and treated (asymptomatic and deformed) embryos for scanning electron microscopy.

The cartilage samples taken from the treated animals showed degeneration of collagen structure and chondrocytes at a high insecticide concentration (4.0%), whereas only retrogression of collagen substance was observed at a low concentration (0.4%). The results explain the development of kyphoscoliosis, cartilaginous fusion of vertebrae, and lordoscoliosis.

Keywords: Chick, cartilage, embryo, scanning electron microscopy.

In the last few years marked progress has been made in the development of methods for evaluating the teratogenic potential of pesticide chemicals. Since the tests have been made for teratogenicity on laboratory rodents, little information has been available on the influence of pesticides on birds (non-target organisms), despite the fact that their effect has great ecotoxicological importance. The principal aim of our investigations was to obtain new information on the toxicological aspects of the commonly used formulation, Wofatox 50 EC.

Organophosphate insecticides cause teratism in chick embryos at concentrations applied in plant protection (Mosconi et al., 1977; Misawa et al., 1982). The results of treatments at the early phase of incubation are kyphoscoliosis, lordoscoliosis, cartilaginous fusion of vertebrae, retardation of tibia and metatarsus, and symptoms of cyllosis (Lutz and Lutz-Ostertag, 1972; Meiniel 1981; Misawa et al., 1982).

As the earlier biochemical examinations of the bone did not show any connection with these alterations, the cervical, macroscopical malformations were explained by an atrophy of the m. longus colli (Meiniel, 1974, 1977; Várnagy et al., 1982).

In the present study the cartilages of vertebrae and phalanges were investigated by scanning electron microscopy (SEM) to reveal the relationships between the toxic effect of insecticide and skeletal deformities.

Materials and methods

Materials

Wofatox 50 EC (active substance: 50% methyl-parathion: 0,0-dimethyl-0/4-nitrophenyl/phosphorothionate); VEB Chemiekombinat, Bitterfeld, GDR. Positive control material: Parathion 20 WP (active substance: 20% parathion: 0,0-diethyl-0/4-nitro-phenyl/phosphorothionate); Bayer AG, Leverkusen, FRG.

Organism

Candled, fertile hen's eggs originating from Shaver Starcross hens were used. After candling, the eggs were incubated in a Ragus incubator as specified in the instructions.

The test materials were used in aqueous suspensions or emulsions at two different dose levels: the lower level corresponded to the concentration applied in the plant protection practice.

Treatment

The eggs were immersed in the suspension, emulsion or distilled water for 30 min at 20 °C (Table I).

Table I

Design of the experiment

Test materials and concentrations	Treatment performed on incubation day	Number of fertile eggs
Control	0	14
(distilled water)	4	13
Wofatox 50 EC	0	13
0.4%	4	13
Wofatox 50 EC	0	12
4.0%	4	13
Parathion 20 WP	0	14
0.3%	4	14

Sampling took place on day 19 of incubation in each case

Table II

Macroscopic and SEM data of the experiment

Test materials and concentrations	Data of the observations			Treatment on incubation day	No. of damaged cartilage	
	Surviving embryos	No. of deformities	Type of malformation		vertebrae	phalanges
Control	13	0	—	0	0	0
(distilled water)	12	3	cyllosis	4	0	1
Wofatox 50 EC 0.4%	4	4	cyllosis, vertebral damage	0	0	1
	9	4	cyllosis, vertebral damage	4	0	2
Wofatox 50 EC 4.0%	9	3	cyllosis, vertebral damage	0	2	2
	7	0	—	4	2	3
Parathion 20 WP 0.3%	14	6	cyllosis, vertebral damage	0	3	2
	14	1	cyllosis, vertebral damage	4	3	3

Sampling

For treatment with each dose, three cartilage samples were taken from the vertebrae (C_{1-7}) and phalanges (P_{1-11}) of the control and treated (asymptomatic and malformed) fetuses at random. The SEM preparation was made by the Milloning (1962) method.

After dehydration the samples were sticked on blocks by bilateral adhesive and steamed with auric-platinum. The SEM examinations were performed with a JEOL JSM 50 A type apparatus.

Results

The macroscopic and SEM observations are summarized in Table II.

Phalanges

The cartilage samples taken from the control birds show a smooth surface. The cartilage cells are slightly convex in the matrix (Fig. 1). No lesions can be observed on the articular head.

A sample from the group treated with 0.4% emulsion of Wofatox 50 EC is shown in Fig. 2. The eggs were treated on day 4 of incubation. The cartilage membrane is absent, the chondrocytes resemble white foci. These changes make deep fissures in the matrix, creating a possibility for further degeneration.

In Fig. 3 there is a sample from the group treated with a 4.0 per cent emulsion of Wofatox 50 EC. The destruction of collagen structure is in an advanced stage. Cartilage cells show nodulous formation and condensation, with deep and large cavities beside them.

Vertebrae

The sample taken from the cervical vertebral body of a control fetus is shown in Fig. 4. The surface is similar to the control phalanx cartilage. The chondrocytes can be seen as slight oval protuberances.

In Fig. 5, there is a cartilage detail from the vertebral arch of the first cervical vertebra from the group treated with a 0.3 per cent suspension of Parathion 20 WP. The treatment was carried out on the first day of incubation. The fetus does not show any gross malformation. There are lacunae in the place of cartilage cells, amorphous connective tissue and large cavities.

Figure 6 shows a microphotograph of the vertebral body from the group treated with a 0.3% suspension of Parathion 20 WP. There is cellular debris on the surface of cartilage.

Discussion

Both organophosphorous chemicals used by us cause the same gross malformations (kyphoscoliosis, lordoscoliosis, cartilaginous fusion of vertebrae, retardation of tibia and metatarsus, cyllosis) in chick embryos.

Parathion 20 WP causes grave microscopic changes of cartilage at 0.3% concentration. The histologically demonstrable cartilage damage depended on the dose of Wofatox EC 50 applied in the experiment. The predominant symptoms and cartilage degeneration do not depend on the day of treatment.

Therefore, the atrophy of skeletal muscles is not the only factor causing teratogenic alteration in the skeleton. These ultrastructural changes of cartilage explain the cervical adhesion, ankylosis and lordoscoliosis of vertebrae.

Although the experiment was based on a practical method and on pesticide concentrations applied in the plant protection practice, articular malformations occurred frequently.

The examination of asymptomatic fetuses especially emphasizes the importance of environmental hazards associated with pesticide use. It demonstrates that the same cartilage deformation occurs in these fetuses as in malformed chickens.

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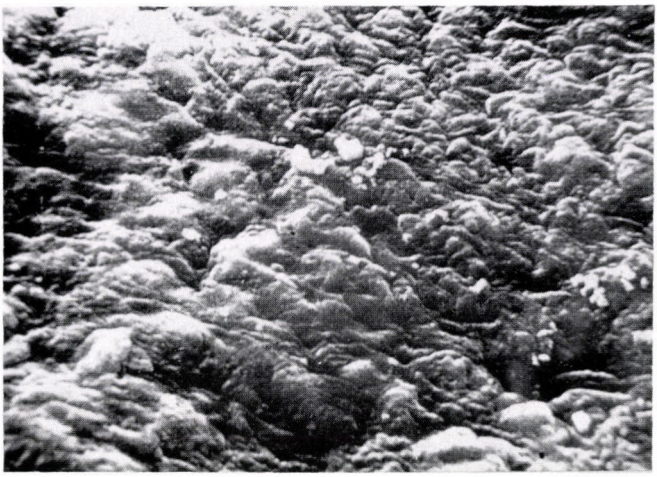


Fig. 1. Articular head from phalanx of control chicken embryo. For explanation, see the text. ×1000

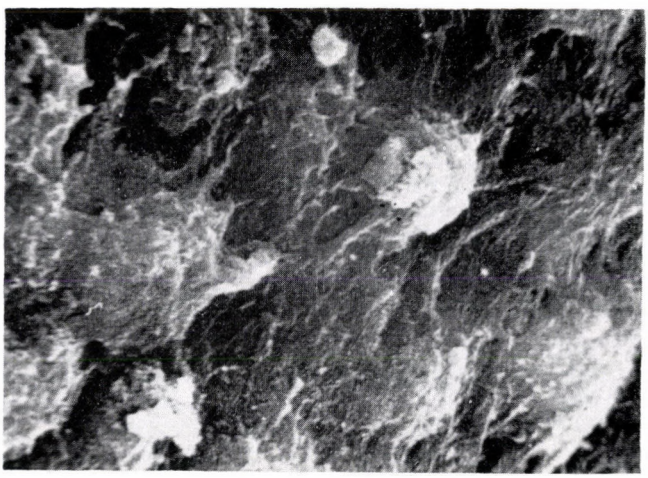


Fig. 2. Cartilage detail from an embryo treated with 0.4% emulsion of Wofatox 50 EC. For explanation, see the text. ×3000

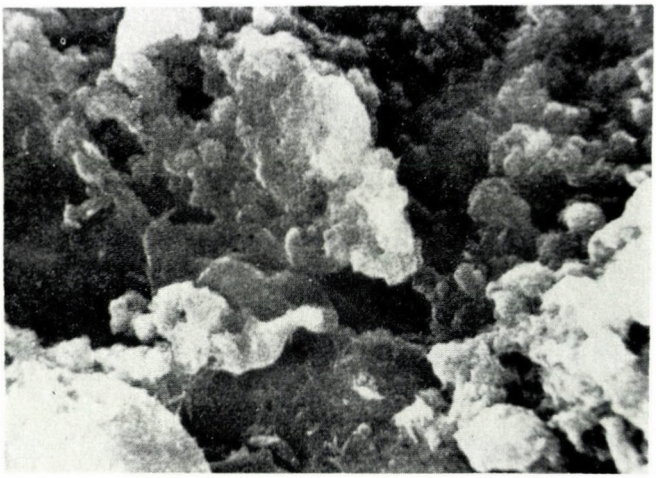


Fig. 3. Sample from the group treated with 4.0% emulsion of Wofatox 50 EC. ×3000



Fig. 4. Control sample from cervical vertebral body. ×1000

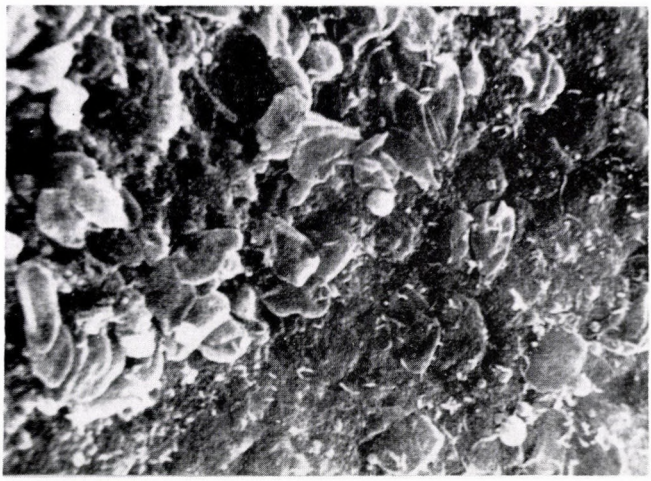


Fig. 5. Microphotograph of vertebral arch from the group treated with 0.3% suspension of Parathion 20 WP. ×1000

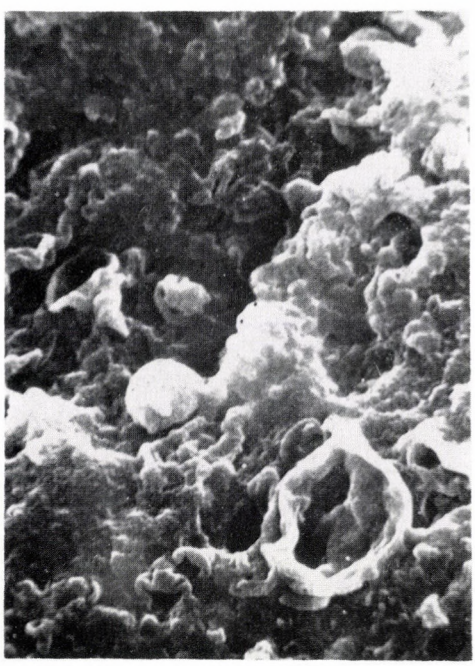


Fig. 6. Cartilage detail from the vertebral body from the group treated with 0.3% suspension of Parathion 20 WP. ×3000

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A SANDWICH ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA) FOR MEASURING ANTIGEN CONCENTRATIONS OF INFECTIOUS BURSAL DISEASE VIRUS

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A double antibody sandwich ELISA was developed for measuring infectious bursal disease virus (IBDV) antigen concentration, primarily for monitoring virus growth during the process of vaccine production. The antibody used for detection was purified by affinity chromatography; thus, most of the background reactions were eliminated. The threshold of detectability is around 10^4 $TCID_{50}$/0.1 ml. The method is well suited for monitoring virus purification and concentration processes, too; it is rapid and simple. The results obtained by the sandwich ELISA were checked by virus titration in cell culture: a fairly good agreement was found.

Keywords: Infectious bursal disease virus (IBDV), antigen detection, sandwich ELISA, virus propagation, virus purification.

Determination of infective virus titre by calculating the 50% end-point (ID_{50}) is the reference method of measuring the concentration of infectious bursal disease virus (IBDV). The main disadvantage of the method is, in addition to some minor drawbacks, that it is time consuming. The aim of the present work was to develop a rapid labour-saving method which does not take more than 24 hours to carry out and is sensitive and specific enough to be used in monitoring virus growth during vaccine production and in examining large numbers of samples.

Double-antibody sandwich ELISA is widely used for diagnostic purposes (Morgan and Smith, 1984; Scherrer and Bernard, 1977; Crittenden et al., 1984; Voller et al., 1976; Korec et al., 1984). It has been used for detecting IBDV and other birnaviruses (e.g. infectious pancreatic necrosis virus of fish) in animal organs (Hattori et al., 1984). However, the procedures developed for detecting virus in biological samples are not sensitive enough for our purposes: the virus to be detected must be concentrated (Nicholas and Thornton, 1984) or propagated (Edevag and Granström, 1986) before applied. Alternatively (Shane et al., 1984), sensitivity can be enhanced by using subunit antigens. There are only few data in the literature on direct virus detection in tissue culture harvests by ELISA without any pretreatment (Ouldridge et al., 1984; Edevag et al., 1985; Schorlemer et al., 1986). The confounding reactions between the two antibody layers and between additives present in the medium

and antibodies bound to them can be eliminated by purifying the indicator antiserum by affinity chromatography (Johansson and Espark, 1978; Forman, 1985; Niklasson et al., 1984). We increased the sensitivity of the method developed for the detection of IBDV antibodies in this way. In addition to monitoring virus growth, the method has proved suitable for checking certain laboratory procedures (e.g. virus concentration, column chromatography, etc.).

Materials and methods

Purification of IBD virus from infected bursa of Fabricius

To obtain purified inocula necessary to produce antisera, 3 weeks old SPF chickens were infected with IBDV (strain Vielitz). After the clinical signs of disease had appeared, the bursae were removed, homogenized in PBS and centrifuged with 8000 *g* for 15 min. The pellet was resuspended in 20 ml PBS, homogenized and sedimented as above. This step was repeated once again. The pooled supernatant fluids were treated three times with freon 113 and, finally, the pooled freon phases were shaken with PBS. From the aqueous phase thus obtained the virus was pelleted by ultracentrifugation (HITACHI SCP85H ultracentrifuge, SRP 28SA rotor) with 131,000 *g* for 120 min. The pellet was resuspended in 0.2 M Tris-HCl buffer (pH 7.4) and sedimented by ultracentrifugation as above, below a 30% sucrose cushion for 5 h. The pellet was dissolved in the same buffer. The material thus obtained contained 8.6 mg/ml protein. It was checked for purity by SDS-polyacrylamide gel electrophoresis (Jackwood et al., 1984; Fahey et al., 1985). The antigen proved good for anti-IBDV antibody determination by either ELISA (Howie and Thorsen, 1981; Marquardt et al., 1980) or agar-gel precipitation (AGPT).

The control antigen was prepared from bursae obtained from healthy SPF chickens and treated as above. Both the antigen and the control antigen were treated with 0.2% ethylene imine for 24 h at 37 °C and mixed with an equal volume of Freund's incomplete adjuvant. The protein concentration of the final vaccine was 50 μg/ml.

Anti-IBDV sera

Five 8 weeks old cockerels, and five Californian White rabbits weighing 2.5 kg, were immunized intramuscularly with 1.0 ml of the vaccine each. Immunization was repeated, with 0.5 ml doses, at two-week intervals. Antibody responses were monitored by ELISA. The animals were killed by bleeding after 8 weeks. The control vaccine was used in a similar manner.

Preparation of IgG fraction from anti-IBDV serum raised in chickens

To 100 ml chicken serum 18 g sodium sulphate was added at a rate to let the salt fully dissolve. After stirring for 1 h, the mixture was centrifuged with 6000 *g* for 20 min and the precipitate was dissolved in 50 ml saline. This step was repeated three times and the solution was dialysed against 4×2 l PBS. After adding 9% sodium sulphate the precipitate was pelleted and the supernatant was supplemented with sodium sulphate to give 14%. The precipitate separated this time was centrifuged as above, dissolved in 20 ml saline and passed through a Sephacryl S-300 column. The pure IgG fraction thus obtained was checked for homogeneity by immunoelectrophoresis. The material was concentrated by repeated precipitation, dialysed, and stored frozen until used.

Purification of the anti-IBDV rabbit serum by affinity chromatography

Two g of CNBr-activated Sepharose 4B (Pharmacia, Sweden) was left to swell in dilute hydrochloric acid (pH 3.0) for one day at room temperature, washed with the same solution (4×200 ml), and drained dry. Subsequently, 1.5 ml SPF chicken serum diluted in 30 ml 0.1 M carbonate buffer (pH 8.3) containing 0.5 M NaCl was applied on it and agitated for 2 h at room temperature.

The gel (volume: 5.2 ml) was filled into a 1×10 cm column. Forty-three mg of protein was bound. After washing with 100 ml carbonate buffer the column was reacted with 2×5 ml 1 M ethanolamine, and then washed with 25 ml carbonate buffer, 25 ml 3 M ammonium rhodanide (pH 6.0), 25 ml 5 M guanidine-HCl and 25 ml BBS buffer (0.1 M boric acid; 0.15 M NaCl, adjusted to pH 7.4 with 0.1 M NaOH; 0.5% Tween 80). The control bursa homogenate supernatant, purified as described above, was passed through a Sephadex G-25 column (Pharmacia, Sweden) and transferred into carbonate buffer. Seven ml of this solution was added to 2 g of swollen gel. The further steps of the procedure were the same as with the chicken serum.

Prior to use the columns were washed with 10 ml volumes of PBS containing 0.5 M NaCl and 0.05% Tween-20 (PBS-T-N). One ml of the rabbit serum to be absorbed was applied on the bursa homogenate column, drained into the bed with 1.5 ml buffer, left to stand for 3 h at room temperature, and then eluted with 40 ml PBS-T-N. The success of the absorption process was checked by agar-gel precipitation, Mancini's test and indirect ELISA.

Affinity gels were regenerated with 25 ml PBS-T, 25 ml 3 M ammonium rhodanide solution and, after every fifth use, 25 ml 5 M guanidine-HCl solution, and then were washed with 25 ml PBS.

Sandwich ELISA procedure

The *anti-IBDV chicken IgG* was diluted 2000-fold in carbonate-bicarbonate buffer. One hundred μl volumes were measured into the wells of the microtitrator tray, agitated for 3 min, incubated for 1 h at 37 °C, and washed with 200 μl PBS-T per well for 3×3 min.

From the *test samples* 3-fold or, occasionally, 4-fold dilution series were prepared. The initial dilution depended on the expectable virus concentration of the sample. PBS-T-N buffer was used as diluent. If the expected virus concentration was low, to 1 ml sample 0.1 ml PBS containing 5 M NaCl and 0.5% Tween 20 was added: thus, the ionic strength and detergent concentration necessary for suppressing nonspecific adsorption could be reached without excessive dilution of the sample.

Two parallel samples (100 μl each) were measured into the wells of the microtitrator tray, agitated for 3 min, incubated for 1 h at 37 °C, then the trays were washed as described above.

The *rabbit serum purified by affinity chromatography* was diluted four-fold in PBS-T-N containing 1% bovine serum albumin (this corresponds to a 160-fold dilution of the undiluted serum). One hundred μl volumes of this dilution were added to the wells, incubated for 60 min at 37 °C, and washed as described above.

The *conjugate* (anti-rabbit IgG-horseradish peroxidase (HRPO), Institut Pasteur, Paris) was diluted 10,000-fold in PBS-T-N. 100 μl volume of the solution was measured into each well of the microtitrator tray. After incubation for 30 min at 37 °C the tray was washed as above.

As *substrate*, orthophenylene diamine (Reanal, Budapest) was used (34 mg OPD and 20 μl 33% hydrogen peroxide dissolved in 100 ml buffer containing 0.05 M Na_2HPO_4 and 0.025 M citric acid, pH 5.5). Two hundred μl volumes were measured into each well and kept in dark room for 15 min. The reaction was stopped by adding 50 μl 2 M sulphuric acid to each well.

Extinction was read in a Titertek Multiskan reader (Flow Laboratories) at 492 nm.

Virus propagation in Vero cells. Cell cultures were prepared in 2 l Roux flasks in MEM-H medium supplemented with 0.4 M glutamine and 8% calf serum. On day 3, the medium of the flasks having a confluent monolayer was replaced with serum-free maintenance medium. To 200 ml medium 1 ml virus inoculum (attenuated IBDV strain GP, $10^{6.5}$ $TCID_{50}$/0.1 ml) was added in different dilutions. Virus growth was monitored for 6 days. The cytopathic effect (CPE) was calculated by Kaerber's method.

Virus purification by column chromatography

A 0.9×54 cm column was packed with Sephacryl S-1000 (Pharmacia, Sweden). PBS containing 1 M NaCl was used as eluent. The flow rate was 0.2 ml/min. The test sample was IBDV grown in Vero cells and propagated on microcarrier ($10^{7.5}$ $TCID_{50}$/0.1 ml). One ml of the sample was applied on the column. The tubes were changed after every 10th drop, and the virus content and protein concentration of each fraction was measured by sandwich ELISA and Folin's reaction, respectively.

Virus purification by ultrafiltration

A Millipore 2 GPM instrument was used with a PTMK filter cassette (limit of transmission: 300,000 daltons). The virus to be purified was 750 ml IBDV; it was propagated as above ($10^{6.5}$ $TCID_{50}$/0.1 ml), and complemented with saline to give 2 l. The instrument operated at maximum reflux until the remaining solution reached 300 ml. Then the sample was again filled up to 2 l, and this step was repeated nine times altogether. On each occasion both the effluent phase and the remaining phase diluted to the initial volume were sampled. The protein concentration and IBDV content of the samples were determined by Folin's method and sandwich ELISA, respectively.

Results

The most important step in the development of our sandwich ELISA system was the absorption of the indicator antiserum by affinity chromatography. The success of the serum absorption process was checked in several ways. It was found that the reaction between the rabbit serum and the negative chicken serum had actually been eliminated. The results of indirect ELISA, used as control, are shown in Fig. 1. Optimal dilutions of the antibody layers were determined by the usual checkerboard titration (Fig. 2). This time the incubation times were not optimized, as our basic aim was to carry out the measurements in less than one work-day, even if this was, to some extent, to the detriment of sensitivity.

Figure 3 illustrates the ELISA results obtained with the purified virus. The broken line indicates extinctions obtained with the control antigen.

Table I contains the statistical evaluation of a series of measurements in which the virus concentration of 50 cell culture fluids was assessed by infective virus titration and ELISA. Both the propagation and the titration took place in Vero cells. The extinctions obtained in the ELISA were extrapolated to the undiluted state, and the background levels were deducted from each value.

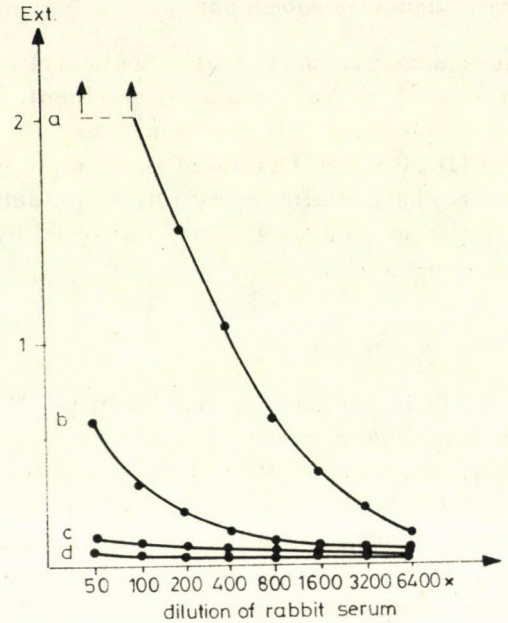


Fig. 1. Indirect ELISA for checking the absorption of rabbit serum. 1st layer: anti-IBDV chicken IgG; 2nd layer: anti-IBDV rabbit serum, (a) untreated; (b) passed through an affinity column made with bursa homogenate: (c) passed through an affinity column made with chicken serum; (d) passed through both columns; 3rd layer: HRPO-labelled anti-rabbit IgG

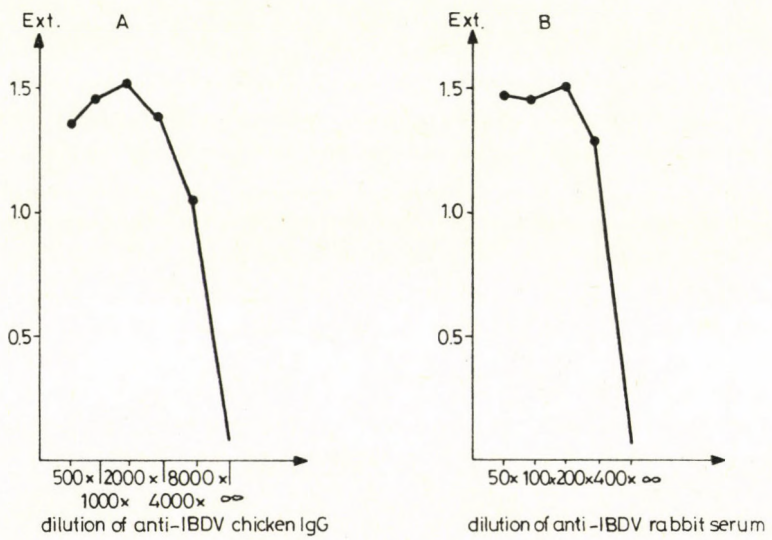


Fig. 2. Optimization of the sandwich ELISA by checker-board titration. (a) the dilution of anti-IBDV chicken IgG is changed; purified virus: 3.5 μg/ml; dilution of anti-IBDV rabbit serum: 200-fold; (b) the dilution of the absorbed anti-IBDV rabbit serum is changed: dilution of anti-IBDV chicken IgG: 2000-fold; purified virus: 3.5 μg/ml

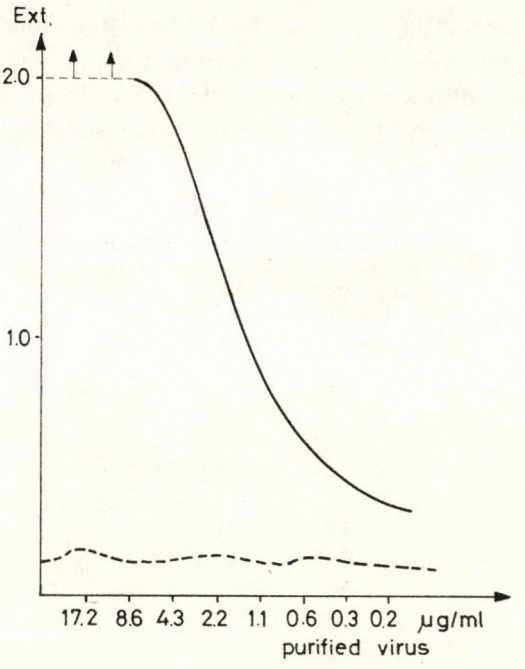


Fig. 3. Sandwich ELISA of IBDV purified from chicken bursa (for conditions of the experiment see Materials and methods). Solid line: purified virus; broken line: purified IBDV-negative bursa

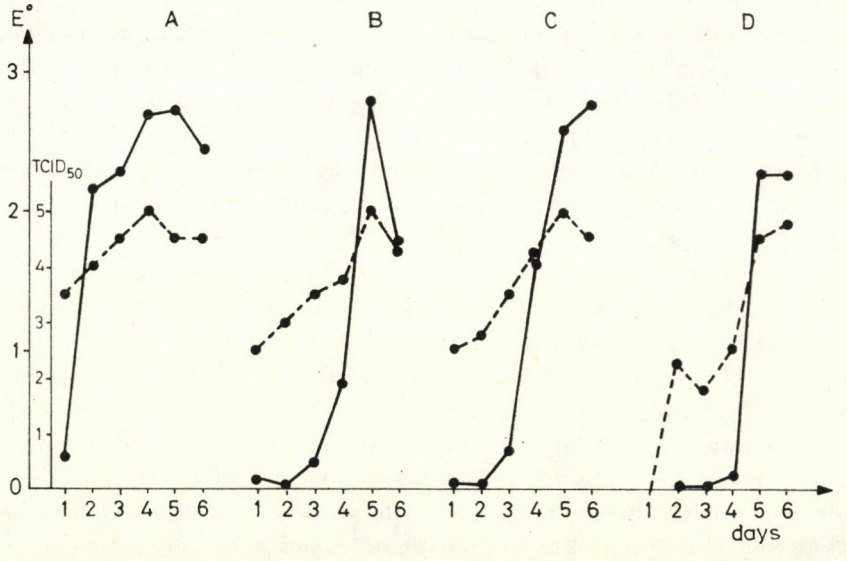


Fig. 4. Propagation of IBDV in Vero cell cultures grown in Roux flasks. Dilution of the virus inoculum: (a) 10-fold; (b) 100-fold; (c) 1000-fold; (d) 10,000-fold. Solid line: ELISA extinction extrapolated to the undiluted state; broken line: infective titre

At titres below $10^{3.5}$ $TCID_{50}$/0.1 ml the ELISA does not give interpretable results. The samples were grouped according to the virus titres, and from the ELISA values group averages were calculated. The coefficient of correlation between the titres and the extinction averages was 0.87. When the data

Table I

Comparison of infective virus titre and ELISA extinction values (E_0) extrapolated to undiluted suspension (IBDV grown in Vero cells)

$TCID_{50}$/0.1 ml (log_{10})	E_0
3.50	0.356 ± 0.252
3.75	1.808 ± 0.804
4.0	1.997 ± 0.985
4.25	2.031 ± 0.689
4.50	2.369 ± 0.335
4.75	2.561 ± 0.589
5.00	2.635 ± 0.312

pair relating to $10^{3.5}$ $TCID_{50}$/0.1 ml (i.e. the threshold of detection) was disregarded, the coefficient of correlation (r) was 0.98.

In the following a few examples are given for possible uses of the measurement procedure developed by us.

The results of virus propagation in Vero cells grown in Roux flasks are shown in Fig. 4. The aim of this experiment was to determine the optimal dilution of the virus inoculum and the optimum incubation time. Virus antigen concentrations were measured by ELISA and the infective virus was titrated in each sample on Vero cells. The ELISA proved less sensitive than titration in cell culture. In our experience, $10^{3.5}$–10^4 $TCID_{50}$/0.1 ml is the threshold of detection for the sandwich ELISA.

Figure 5 shows the results of a gel-chromatographic separation. The test sample was IBDV grown in Vero cells ($10^{7.5}$ $TCID_{50}$/0.1 ml). All fractions were assayed for protein content, then IBDV antigen concentration was determined by ELISA. The results have two interesting features: firstly, two peaks were obtained, suggesting that the intact and injured virions had been separated; however, because of the low concentrations the fractions could not be identified. Secondly, well-detectable virus activities occurred together with extremely low protein concentrations much below the threshold of detectability, indicating that the gel-chromatographic purification had been very efficient.

A process of virus purification by ultrafiltration is illustrated in Fig. 6. The initial suspension was IBDV ($10^{6.5}$ $TCID_{50}$/0.1 ml) grown in Vero cell cul-

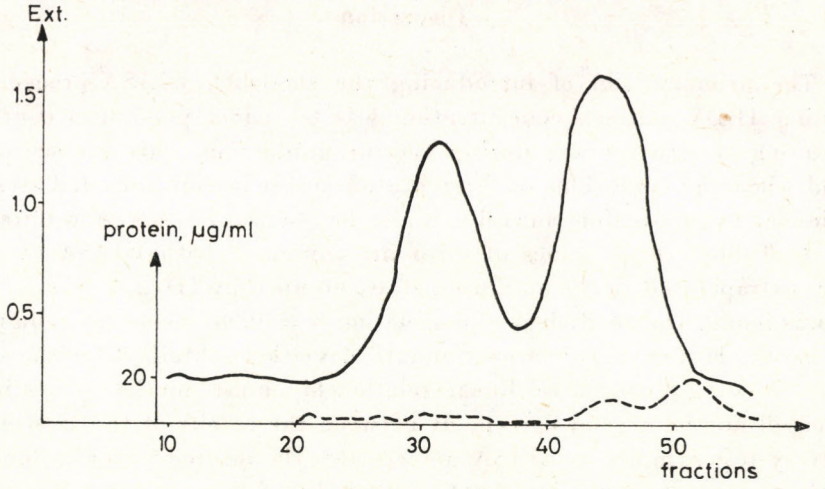


Fig. 5. Detection of IBDV separated by column chromatography. Column: Sephacryl S-1000, 0.9×54 cm; initial virus titre: $10^{7.5}$ $TCID_{50}$/0.1 ml. Solid line: ELISA extinction values; broken line: total protein concentration

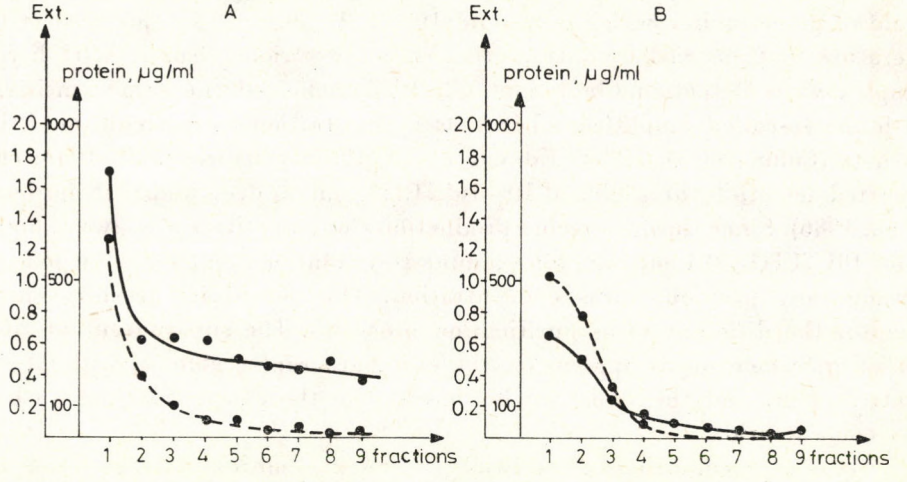


Fig. 6. Virus purification with a Millipore 2 GPM virus-concentrating apparatus. (a) initial solution; (b) effluent (solution passing through the filter). Solid line: ELISA extinction values; broken line: total protein concentration

tures. In theory, the transmission limit of the applied filter was 300,000 daltons. Despite this fact, well-detectable virus antigen activity was found in the filtrated fractions. Supposedly this might have been due to separation of the intact and injured virions, or viral debris. The confirmation of this hypothesis, however, warrants further studies.

Discussion

The primary aim of introducing the sandwich ELISA procedure for assessing IBDV antigen concentration was to render possible a continuous monitoring of virus growth during vaccine production. This is essential in a period when the conditions of fermentation are being optimized. Later on, a continuous in-production control can also be realized by this procedure.

In Table I ID_{50} values of virus are compared with ELISA extinction values extrapolated to the undiluted state, grouped by ID_{50}. A strong correlation was found; the coefficient of correlation ($r = 0.98$) meets the requirement of linearity. However, the large standard deviations obtained for the various groups show that this close linear relationship must not be overestimated. There will not be a good agreement between the results of the two methods for every test sample, as ELISA detects also the injured virions which have lost their ability to multiply (Clayton et al., 1986).

As it has been pointed out above, in sensitivity the sandwich ELISA is inferior to the classical virus titration. Under practical conditions this means that the titre of the inoculum and the antigen concentrations measured on the first and, occasionally, second day of virus propagation remain below the threshold of detectability which is around 10^4 $TCID_{50}$/0.1 ml. In this respect the literature contains rather contradictory data for various viruses. With herpes simplex virus detection Clayton et al. (1986) achieved the same sensitivity with the so-called amplified ELISA as with titration in cell culture. Other authors (Collins et al., 1985; Edevag et al., 1985; Hattori et al., 1984; etc.) reported detection thresholds of 10^3–10^4 $TCID_{50}$/ml, or even higher (Schorlemer et al., 1986). Since during vaccine production the virus titres are always higher than 10^4 $TCID_{50}$/0.1 ml, we can examine test solutions and virus suspensions without any previous virus concentration. This sensitivity is sufficient to monitor the different virus purification processes. The supernatant of Roux flasks or fermentors is applied on the chromatographic column without any pretreatment, and the virus can be detected in the eluate fractions without any concentration.

It is a precondition of sandwich ELISA procedures that the lower and upper antibody layers do not react with each other or with contaminations present in the samples under testing (Hattori et al., 1984). Since IBDV is usually grown in cell cultures of chicken origin under both laboratory and industrial conditions, the antisera raised in rabbits had to be freed of antibody populations produced against contaminations present in the virus purified from chicken bursae. In this way the nonspecific reaction caused by different contaminants present in the test virus suspension was also eliminated. Thus, the virus concentration of any medium became measurable. At the same time, the background level attributable to the reaction of anti-IBDV chicken IgG

and anti-IBDV rabbit serum decreased to a minimum. It should be stressed that by using monoclonal antibodies the above aim is attained without any intervention. Unfortunately, for the time being monoclonal antibodies are still not available to many laboratories (De Boer and Osterhaus, 1985; Collins et al., 1985; Crouch et al., 1984; Heinz et al., 1986; Forman, 1985).

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BOOK REVIEW

Pathogenesis of Bacterial Infections in Animals. Edited by Carlton L. GYLES and Charles O. THOEN (twenty-two contributors). Iowa State University Press, Ames, Iowa, USA. First edition, 1986. pp. 227, in English.

Knowledge on the pathogenesis of bacterial diseases has increased enormously in recent years as a result of advances in molecular biology and biochemistry in general and in molecular genetics in particular. Bacteria were the first which were studied in this field of science. These findings improved our knowledge of host–parasite relationships at tissue, cell and molecular levels. Chapters of this book provide a good insight into these principles. Researchers recognized, among others, that bacteria are able to extract iron from host tissues and to grow under iron-limiting conditions, and this is highly correlated with virulence, they can attach to host cells, have strategies to combat mucosal immunity, are able to evade phagocytosis and to survive in phagocytic cells; they may degrade immunoglobulins (IgA, IgG), which is a fascinating potential virulence factor. It turned out that some diseases may be attributable to a single toxin. Development in separation methods has resulted in purification and characterization of several bacterial products related to pathogenesis.

Progress in understanding the pathogenesis of bacterial infections in animals, which has occurred as a result of applications of these advances, is reviewed in this book. This is the first edition of the book. The editors' aim, they write, was to fill a void in the current literature on mechanism of bacterial diseases. They claim: pathogenesis is the crossroads at which microbiologists, pathologists and clinicians find common ground. Their book is intended to be useful for students of these disciplines.

The intention of the editors was a worthy one: the book merits careful reading.

The book is divided into 25 chapters, printed from a camera-ready copy: this has some effect on the quality of photographs. It was written by 22 authors. Eight authors are from the University of Guelph (Department of Microbiology), a fact reflecting the high standard of work done in that Department.

Any assessment of the quality of a chapter must inevitably be subjective. Some contributions are outstanding: Streptococcus, Mycobacterium, Salmonella. In contrast, some chapters are a little disappointing. Why was it felt necessary to devote so many pages to *Escherichia coli*, by far the largest allowance in the book? As with all multi-author textbooks, a wide range of styles has been adopted. Moreover, the information provided in different chapters ranges from the pragmatic description of the pathogenesis to sections of pathogenesis plus sources of infection, drug resistance, control and treatment. Chapters have been included which cover both the animal and human diseases, an excellent concept. Some additional comments: (a) contributions are headed by bacterial names only. It would have been better, where appropriate, to mention the name of the particular disease under the heading. The reader, in some cases, has to look for the name of the disease hidden in the text. (b) Chapter Shigella is included in the book, but *Francisella tularensis* (tularaemia) is not. Shigella causes dysentery only in primates; *Francisella tularensis*, first described in North America, in 1912, causes zoonoses of world-wide importance. (c) The species *Treponema hyodysenteriae* should be classified in the order Spirochaetales, not in the group of Gram-negative anaerobes. (d) Related to the pathogenesis, in chapters Clostridium and non-spore-forming anaerobes the following basic facts are missing: anaerobes differ in their sensitivity to oxygen ranging from those which are just barely anaerobes, such as *Clostridium perfringens*, through moderate anaerobes, capable of growing in the presence of up to 2 to 5 per cent oxygen, to strict or obligate anaerobes, such as most Gram-negative anaerobes, incapable of

growth if there is more than 0.5 per cent oxygen present. (e) Only attenuated Mycoplasma vaccine against CBPP (KH_3J, T_1) has proved its efficacy.

Selected references to the literature are included in each chapter. Unfortunately, many references are to American journals and textbooks; European and Japanese authors are not mentioned very often.

Notwithstanding the above criticism, this book would be a useful addition to any institute or private library, and would also be of value to veterinary undergraduates. It fits nicely between the extensive references textbooks. Information on recent advances in pathogenesis is scattered in several journals (biochemical, immunological, etc.): it is good to find them together in this well-written book, which, it is to be hoped, will regularly be followed by new editions. It is not, however, a comprehensive text covering all aspects (epidemiology, differential diagnosis, etc.) of bacterial infections in animals, and would not, by itself, serve as a general guide to veterinary microbiology and infectious diseases.

L. Pesti

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COMING EVENT

The 18th Congress of the German Veterinary Medical Society will be held from 5–8 April 1989 in Bad Nauheim (near Frankfurt/Main), Federal Republic of Germany. The topic is "Pathogenesis and Control of Factor Diseases". The papers should not only consider infectious but also non-infectious factor diseases.

Papers can be presented in German or English. The title of papers should be submitted until 1 June 1988 to the President of the DVG, Prof. Dr. Dr. h.c. mult. Anton Mayr, Veterinärstrasse 13, D-8000 München 22, Federal Republic of Germany.

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MANUSCRIPT STYLE

Manuscripts must be in English or Hungarian and clearly and concisely written. They should be typed double spaced with wide margins. Two copies of the manuscript should be submitted.

FORM OF MANUSCRIPT

Title. The title should be a clear and concise statement of the contents in not more than 14 words. A short running title of not more than 40 letters should also be supplied. This is followed by the authors' initials (full first name of women) and surname, and the name of the institution where the work was done. The mailing address of the authors must also be indicated here.

Abstract. This should not exceed 200 words and should outline briefly the purpose of the study and detail important findings and the authors' principal conclusions. Redundant phrases, generally known information and repetition should be avoided.

Introduction. This part should state briefly the nature and purpose of the work and cite recent important work by others.

Materials and methods. Describe materials, methods, apparatus, experimental procedure and statistical methods in sufficient detail to allow other authors to reproduce the results This part may have subheadings.

Results. The experimental data should be presented clearly and concisely. Avoid repeating information presented in tables and figures.

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Acknowledgement of grants and technical help.

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EFFECT OF SOIL FERTILITY ON THE AMINO ACIDS AND NUTRITIONAL VALUE OF WHEAT GRAIN

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(Received January 13, 1988)

The effects of soil nitrogen level on the amino acid composition and nutritive value of two samples of wheat were studied. The two samples were collected from the same line and grown. Different levels of nitrogen (N) fertilizer were applied. The sample receiving the higher level of N showed a greater increase in crude protein (CP; $P < 0.05$); at the same time its relative amount of threonine and lysine was lower ($P < 0.05$), in spite of its higher total amino acid content.

Feeding trials with broiler chickens indicated that birds fed on wheat grown at a lower level of N fertilizer had a higher body weight ($P < 0.05$) and growth rate ($P < 0.01$). It is suggested that the use of high-protein wheat might cause some nutritional problems due to amino acid imbalances in the diet.

Keywords: Soil fertility, nitrogen, fertilizer, wheat, amino acids, nutritional value, broiler chicken

Recently a higher per area food production has become an objective of prime importance. To achieve this, the application of fertilizers has to be improved. Parallel with the increase in wheat yield, its nutritional value as a food or feed, and how it is influenced by fertilizers, is acquiring greater importance. In the last few years attention has been focussed on genetic variations and environmental effects on the levels of nitrogen compounds in wheat.

Numerous investigations have shown that nitrogen fertilizers, while increasing the yield and the percentage of protein in wheat grain, also affect its amino acid composition and protein quality. Although it is well established that there is a negative relationship between the protein content and a number of essential amino acids, notably lysine, in wheat grain (Byers et al., 1978; Eppendorfer, 1978; Johnson et al., 1972), little attention has been given to the effects of soil fertility on the biological value of protein.

The objective of this investigation was to determine the effects of soil nitrogen (N) level on the amino acid composition of wheat samples differing in crude protein (CP) content and the nutritional value of these wheat samples in chicken diets.

Materials and methods

Two wheat samples, grown in fertilization experiments, were analysed. The experiments were conducted within the framework of a government-level research programme called "Studies on the Effects of Various Amounts and Rates of Applying Fertilizers". The wheat samples to be tested were selected arbitrarily on the basis of protein content. The line studied was MV-4 grown in the sequence of winter wheat–corn–corn–winter wheat in 1984.

The applied amounts of fertilizers were 50, 50 and 100 (wheat 1) and 150, 50 and 100 (wheat 2) kg of nitrogen (N), phosphorus (P) and potassium (K) per hectare, respectively. The total amount of P and K fertilizers and half of the N fertilizer were applied in the autumn, whereas the other half of N in the spring. The wheat was harvested at physiological maturity. Samples were pooled by treatment from replications.

Protein was determined by Kjeldahl analysis on each sample and adjusted to a 12% moisture basis. The amino acids were analysed by ion-exchange chromatography using a BIOTRONIK LC 5001 analyser. Calculations of the essential amino acid index (EAAI; Oser, 1951) and of the chemical score (CS Mitchell and Block, 1946) were made as an evaluation of optimal amino acid composition.

Each pooled sample of wheat was incorporated into a wheat–soybean meal diet (Table I). Diets were formulated to be isonitrogenous by adding starch to treatment 2 so that the proportions of nitrogen supplied by wheat and soybean meal were identical in diets 1 and 2. These diets only marginally sup-

Table I

Composition of diets

Ingredient	Treatment	
	1	2
	%	
Wheat (1)	72.60	—
Wheat (2)	—	63.05
Soybean meal 48.5%	24.00	24.00
Starch	—	9.55
AP-17	0.40	0.40
Sunflower oil	1.00	1.00
NaCl	0.50	0.50
$CaCO_3$	1.00	1.00
Premix	0.50	0.50
Calculated nutrients		
Crude protein%	20.48	20.48
AME MJ/KG	11.81	12.07
Ca%	1.19	1.19
P%	0.45	0.45

Table II

Amino acid contents of wheat samples in dry matter

Item n = 6	Treatment 1 ($\bar{x} \pm S$)		Treatment 2 ($\bar{x} \pm S$)		2/1×100	LSD (P < 0.05)
Aspartic acid	0.490	0.039	0.507	0.029 NS	103	
Threonine	0.267	0.020	0.255	0.018+	96	0.01
Serine	0.458	0.033	0.482	0.025*	105	0.02
Glutamic acid	3.143	0.224	3.508	0.152**	112	0.21
Proline	1.033	0.046	1.158	0.046+	112	0.12
Glycine	0.382	0.026	0.388	0.022 NS	102	
Alanine	0.367	0.023	0.387	0.015*	105	0.01
Cysteine	0.167	0.110	0.183	0.019*	110	0.01
Valine	0.382	0.023	0.413	0.018**	108	0.01
Methionine	0.097	0.022	0.120	0.044 NS	124	
Isoleucine	0.290	0.013	0.313	0.008**	108	0.01
Leucine	0.643	0.041	0.683	0.027*	106	0.03
Tyrosine	0.207	0.012	0.215	0.008 NS	104	
Phenylalanine	0.407	0.025	0.435	0.022*	107	0.02
Histidine	0.262	0.015	0.273	0.010+	104	0.01
Lysine	0.247	0.015	0.245	0.016 NS	99	
Arginine	0.417	0.030	0.425	0.023 NS	102	

NS = Not significant; + = $P < 0.1$; * = $P < 0.05$; ** = $P < 0.01$

Table III

Amino acid contents of wheat samples as % of crude protein

Item n = 6	Treatment 1		Treatment 2		LSD P = 0.05
	$\bar{x}$	S	$\bar{x}$	S	
Aspartic acid	4.03	0.32	3.61	0.21*	0.33
Threonine	2.19	0.16	1.82	0.13**	0.21
Serine	3.76	0.27	3.44	0.18*	0.29
Glutamic acid	25.80	1.84	25.02	1.09 NS	
Proline	8.50	0.30	8.26	0.33 NS	
Glycine	3.14	0.22	2.77	0.15**	0.22
Alanine	3.01	0.19	2.76	0.11*	0.18
Cysteine	1.37	0.09	1.31	0.13 NS	
Valine	3.14	0.19	2.95	0.13+	0.19
Methionine	0.80	0.18	0.86	0.31 NS	
Isoleucine	2.37	0.11	2.23	0.06*	0.09
Leucine	5.28	0.34	4.87	0.19*	0.29
Tyrosine	1.70	0.10	1.54	0.06**	0.10
Phenylalanine	3.34	0.20	3.10	0.16+	0.24
Histidine	2.15	0.12	1.95	0.08**	0.10
Lysine	2.03	0.12	1.75	0.12**	0.17
Arginine	3.41	0.25	3.03	0.17**	0.22

NS = Not significant; + = $P < 0.1$; * = $P < 0.05$; ** = $P < 0.01$

plied the chicks' methionine requirement. Each diet was fed to ten replicate pens of 10 one-day-old, male chicks (Bábolna Tetra-B) over a period of 21 days. The chicks were kept in tiered batteries and were allowed to consume feed and water ad libitum. The pens and treatments were randomly assigned to batteries. A biological protein evaluation was carried out by determining the protein efficiency ratio (PER; A. O. A. C., 1975).

The data were analysed as a completely randomised design with the pen considered the experimental unit, using analysis of variance (Sváb, 1969).

Results and discussion

The crude protein content of wheat 2 increased by 14% ($P < 0.05$) as compared with wheat 1 (from 13.7% to 15.9%) as the dose of N fertilizer was increased from 50 to 150 kg N per hectare. The amino acid contents of wheat

Table IV

Chemical evaluation of protein in wheat samples

n = 6	Treatment		2/1×100	
	1	2		
Non-essential amino acids*				
Aspartic acid	4.03	3.61		90
Serine	3.76	3.44		91
Glutamic acid	25.80	25.02		97
Proline	8.50	8.26		97
Alanine	3.01	2.76		92
Tyrosine	1.70	1.54		91
Total:	46.80	44.63	Average:	93
Essential amino acids*				
Threonine	2.19	1.82		83
Glycine	3.14	2.77		88
Cysteine	1.37	1.31		96
Valine	3.14	2.95		94
Methionine	0.80	0.86		108
Isoleucine	2.37	2.23		94
Leucine	5.28	4.87		92
Phenylalanine	3.34	3.10		93
Histidine	2.15	1.95		91
Lysine	2.03	1.75		86
Arginine	3.41	3.03		89
Total:	29.22	26.64	Average:	92
E/N	0.62	0.60		97
EAAI	47.38	43.21		91
CS	21.82	22.23		102

* = g/16 g N

Table V

Effects of fertilizers on weight gain and efficiency of feed utilization of chicks

n = 100	Treatment	Body weight g		Weight gain g/day/chick		Feed/gain		PER	
		$\bar{x}$	S	$\bar{x}$	S	$\bar{x}$	S	$\bar{x}$	S
Day-old	1	44	0.71	—		—		—	
age:	2	44	0.97	—		—		—	
Week 1	1	108	4.33	9.3	0.46	2.01	0.22	2.41	0.26
	2	105	3.53	8.6*C	0.45	2.18	0.34	2.39	0.37
Week 2	1	221	12.3	16.1	1.39	2.22	0.23	2.18	0.26
	2	207*A	10.4	14.5*D	1.00	2.34	0.26	2.20	0.22
Week 3	1	385	27.5	29.1	1.62	2.14	0.17	2.25	0.18
	2	348*B	24.0	25.9**E	2.01	2.22	0.24	2.32	0.23
Total:	1	385	27.5	17.5	1.00	2.16	0.14	2.22	0.15
	2	348*B	24.0	15.8**F	1.09	2.25	0.17	2.28	0.16

*A = $P < 0.05$ LSD = 11.2 g
*B = $P < 0.05$ LSD = 26.5 g
*C = $P < 0.05$ LSD = 0.53 g
*D = $P < 0.05$ LSD = 1.27 g
**E = $P < 0.01$ LSD = 2.12 g
**F = $P < 0.01$ LSD = 1.08 g

1 and 2 samples are summarized in Table II as percentages of air-dry matter (12% moisture; absolute amount) and in Table III as percentage of total crude protein (relative amount). The absolute amount of threonine and lysine was somewhat lower in wheat 2 but the total amino acid content was higher as compared to wheat 1.

The relative amount of threonine and lysine decreased by 17% ($P < 0.01$) and 14% ($P < 0.01$), respectively, in wheat 2.

In Table IV, a comparison was made between the protein quality of the samples by chemical evaluation. Almost all of the data indicated a reduced protein quality in wheat 2 as compared to wheat 1. This suggests that the quality of protein was adversely affected by increased N fertilization. These trends are consistent with those reported previously by Eppendorfer (1978).

In the feeding trial shown in Table V, chicks fed wheat 1 had 11% higher body mass ($P < 0.05$) and gained faster ($P < 0.01$) than those fed wheat 2. The feeding of wheat 2 did not improve the performance of chicks. Similar trends were found for feed efficiency and PER values, but the interaction was not significant.

The data indicate that this kind of N fertilization of wheat, while increasing its protein and total amino acid content, has no beneficial effect on its nutritional value for chicks. This must have been due to the fact that higher

N fertilizer application decreased the lysine and threonine contents of wheat protein. The decrease in these two amino acids might have caused the difference in the performance of chickens. It is suggested that the use of high-protein wheat might cause some nutritional problems due to amino acid imbalances in the diet.

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POSTPARTAL OVARIAN FUNCTION IN DAIRY COWS KEPT ON DIFFERENT FEEDING REGIMES

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(Received December 5, 1987)

The characteristics of postpartal ovarian activity were compared in two groups of cows kept on different feeding regimes. The cows had previously calved 3–5 times. The first group was kept on a conventional feeding regime (Group C; n = 39): during the dry period the net energy and crude protein level of the ration considerably exceeded the actual requirements, and after calving it satisfied the requirements of about 30 kg milk production. The feeding regime used in the second group followed the nutrient requirements reported by the NRC (Group NRC; n = 27). At the early stage of lactation it was sufficient to produce about 35 kg of milk. Involution and resumption of ovarian cyclicity were monitored by regularly repeated rectal and vaginal examinations and by the progesterone assay of milk samples collected at 3- to 4-day intervals over a period of 12 to 13 weeks.

In Group C involutional complications occurred more frequently and required longer treatment than in Group NRC. Disorders of the re-establishment of ovarian cyclicity, in which disturbances of the energy balance may have been the underlying cause (e.g. delayed resumption of cyclic ovarian activity, irregular cycles, cessation of cycling), were also more common in Group C. In Group C there was a close (r = 0.582), whereas in Group NRC only a low (r = 0.361) positive correlation between milk production in the period between postpartum days 30 and 60 and the duration of postpartal acyclia.

Keywords: Ovarian activity, post partum, dairy cow, feeding

In non-suckling dairy cows the duration of postpartal acyclia is usually not longer than 28–35 days. Delayed resumption of cyclic ovarian function is an infrequent cause of infertility. No close correlation has been reported between milk production and the frequency of certain ovarian disorders (Bulman and Lamming, 1978; Bulman and Wood, 1980; Ball, 1982; Claus et al., 1983; Pirchner et al., 1983). In Hungarian dairy herds, however, prolonged postpartal acyclia and certain other disorders (irregular cycling, cessation of ovarian cyclicity) have proved to be much more common. In older, high-yielding cows affected by involutional complications many of the above disorders occur in a cumulative manner (Huszenicza et al., 1987). This contradiction between observations made in Western Europe and Hungary is probably due to differences in management technology and feeding.

The aim of the present work was to confirm this assumption and to compare postpartal ovarian functions. The experiment was conducted on dairy cows kept under large-scale conditions and on different feeding regimes (on a

conventional feeding regime, i. e. fed moderately high energy levels during the dry period, and according to the NRC recommendations (1978), i. e. fed low energy levels during the dry period).

Materials and methods

The experiment was conducted in two large-scale dairy herds of more than 1000 Holstein-Friesian and Holstein-Friesian × Hungarian Fleckvieh crossbred cows each, kept under loose housing conditions. Cows calving between 15 February and 10 April for the 3rd–5th time were examined. For the experiment, in the last days of the dry period we selected clinically healthy cows that had produced at least 3500 kg of 40% fat corrected milk (FCM) during their preceding 305-day lactation. Individuals with premature delivery, calving difficulties, twin pregnancy, metabolic disorders or signs pathognomonic to severe organic lesions, as well as cows producing less than 18 kg of FCM daily during the period of study were excluded from the evaluation. In both herds the cows (including those selected for the experiment) were assigned to groups of 80–100 on the basis of milk production. The dry cows were kept in groups of about 50. After calving the animals were transferred to the group of the highest yielding cows, from where they were moved to the cow group corresponding to their actual milk production level at milking-in the following month.

Table I

Quantity and composition of the daily ration in the conventionally fed cows (Group C)

		Corn silage	Crushed corn grain[1]	Alfalfa hay	Meadow hay	Dairy diet[2]	Preparatory diet[2]
		Daily ration, kg/cow					
Groups formed according to milk production	≥30 kg	25	4.5	4	—	6.0	—
	25 kg	25	3.5	4	—	5.0	—
	20 kg	25	2.0	4	—	3.5	—
	≤15 kg	25	1.0	—	4	2.0	—
Dry cows	first 6 weeks	25	—	—	4	—	2.0
	last 2 weeks	20	—	—	5	—	4.0
		Contents					
Dry matter	%	35.5	74.9	90.1	90.3	89.4	89.5
Crude protein	%	3.7	5.3	20.4	11.0	17.9	17.2
Crude fat	%	0.9	3.0	2.5	2.4	2.7	2.9
Crude fibre	%	6.0	2.8	27.3	32.3	NT	NT
N-free extract	%	23.2	63.7	30.7	38.2	NT	NT

[1] Stored wet in bunker silo; [2] formulated according to a receipt of the Agricultural Combinate Bábolna; NT = not tested

Table II

Quantity and composition of the daily ration in cows fed according to the NRC requirements (Group NRC)

		Corn silage	Crushed corn silage[1]	Alfalfa hay	Meadow hay	Dairy diet[2]	Bran[3]
		Daily ration, kg/cow					
Groups formed according to milk production	≥35 kg	15	6.5	7	—	8.5	—
	30 kg	15	5.0	6	—	7.5	—
	25 kg	16	5.5	5	—	6.0	—
	20 kg	15	3.0	4	2	4.7	—
	≤15 kg	18	2.0	—	8	2.0	—
Dry cows	first 6 weeks	15	—	—	4	—	—
	last 2 weeks	12	—	—	4	—	2
		Contents					
Dry matter	%	39.6	62.7	90.0	90.5	89.7	89.1
Crude protein	%	3.7	6.2	21.5	14.0	16.4	16.4
Crude fat	%	1.3	2.1	2.3	2.4	2.6	3.1
Crude fibre	%	8.2	5.7	20.3	28.3	3.7	7.6
N-free extract	%	24.9	47.3	35.3	38.4	60.9	56.7

[1] Prepared with addition of carrot; [2] formulated according to a receipt of the Agricultural Combinate Bábolna; [3] feed yeast content: 3%

In the first herd four production groups were formed in addition to the dry cow group. The ration was formulated from feeds of known content values as recommended by the Hungarian Feed Standardizing Committee in 1980 (conventionally fed animals, Group C; Table I). On the second farm five production groups were formed and the rations were formulated as recommended by the NRC in 1978 (Group NRC; Table II). Chemical analysis of the feeds was performed according to Hungarian Standard no. MSz. 6830-66 and its currently valid modifications (Hungarian Feed Standardizing Committee, 1980). When checking the actual energy content of the ration, the net energy for milk production (NE_1) was calculated by the equation of Moe and Tyrrell (1976) from actual chemical composition and digestibility coefficients given in the Hungarian standard. To permit a correct comparison with the NRC standard, a discount factor of 4 was used consistently for all feeds. The daily energy and protein intakes of an "average cow" of each group were calculated and are shown in Fig. 1 together with the actual requirements (NRC, 1978). Feeds were distributed twice daily. The fermented feeds and concentrates were given as a freshly prepared, uniform mixture, and hay was offered separately. Drinking water, NaCl and mineral supplement were always available ad libitum.

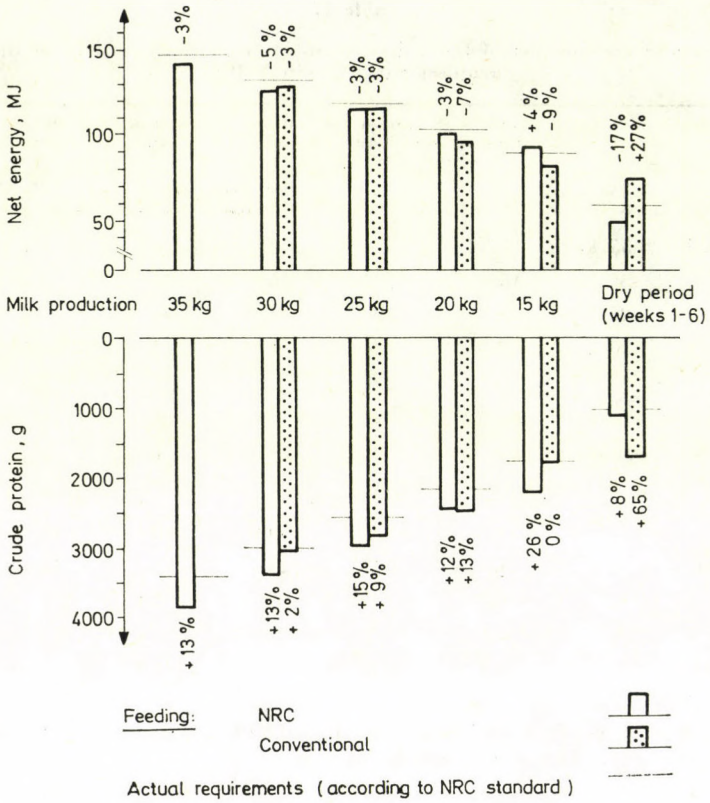


Fig. 1. Energy and protein supply of cows in the two feeding groups

The treatment of involutional disorders and the applied reproduction technology were the same as described previously (Huszenicza et al., 1987).

Over a period of 12 to 13 weeks after calving, at 3- to 4-day intervals milk samples were collected and assayed for progesterone after defatting, without extraction, with an enzyme-immunoassay (Solti et al., 1986). Simultaneously, rectal and vaginal examinations were performed at 7- to 14-day intervals. Reproduction data were recorded up to postpartum day 150. The re-establishment of cyclic ovarian activity was evaluated by comparing the results of the clinical examinations and the progesterone profile. This enabled us to differentiate between cows free from pathological changes and those characterized by ovarian disorders (delayed resumption of cycling, irregular cycles, cessation of cycling, cystic degeneration of follicles, permanent corpus luteum (CL) function). For details, see an earlier paper (Huszenicza et al., 1987).

Results

Sixty-six cows gave interpretable results (39 of them belonged to Group C and 27 to Group NRC). There was no significant difference in milk production between the two groups of approximately identical breed composition and age (milk production in the preceding lactation: 5270 $\pm$ 942 kg vs. 5759 $\pm$ $\pm$ 1242 kg FCM; daily milk production between day 30 and 60 of the lactation studied: 28.8 $\pm$ 4.8 vs. 29.3 $\pm$ 5.7 kg FCM).

Retained placenta and other involutional complications were more frequent in Group C than in Group NRC, in which involutional complications were less severe and required shorter treatment (Table III).

Table III

Involution of cows in the two feeding groups

	Feeding				Level of significance
	Conventional (n = 39 = 100%)		According to the NRC requirements (n = 27 = 100%)		
	n	%	n	%	
Retained placenta	10	25.6	1	3.7	$P < 0.05$
Involution uncomplicated (no therapy required)	10	25.6	12	44.4	
complicated (therapy: <30 days)	4	10.3	9	33.3	$P < 0.05$
complicated (therapy: ≥30 days)	25	64.1	6	22.2	$P < 0.01$

Table IV

Reproductive indices of cows in the two different feeding groups

	Feeding		Level of significance
	Conventional (n = 39 = 100%)	According to the NRC requirements (n = 27 = 100%)	
	days; $\bar{x} \pm SD$		
Postpartal acyclia	39.9 $\pm$ 25.2	29.0 $\pm$ 14.4	$P < 0.05$
Start of first CL phase longer than 10 days	48.3 $\pm$ 25.6	40.0 $\pm$ 19.5	
First insemination[1]	77.5 $\pm$ 30.8 (n = 34)	64.1 $\pm$ 26.2 (n = 27)	
Conception[2]	112.2 $\pm$ 34.7 (n = 24)	95.5 $\pm$ 27.8 (n = 23)	$P < 0.05$

[1,2] Based on data of cows inseminated[1] and conceived[2] within 150 days post partum

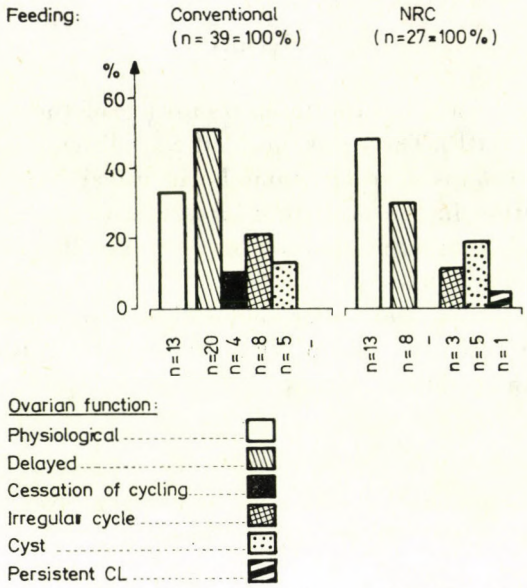


Fig. 2. Incidence of ovarian dysfunction in cows fed conventionally and according to the NRC requirements

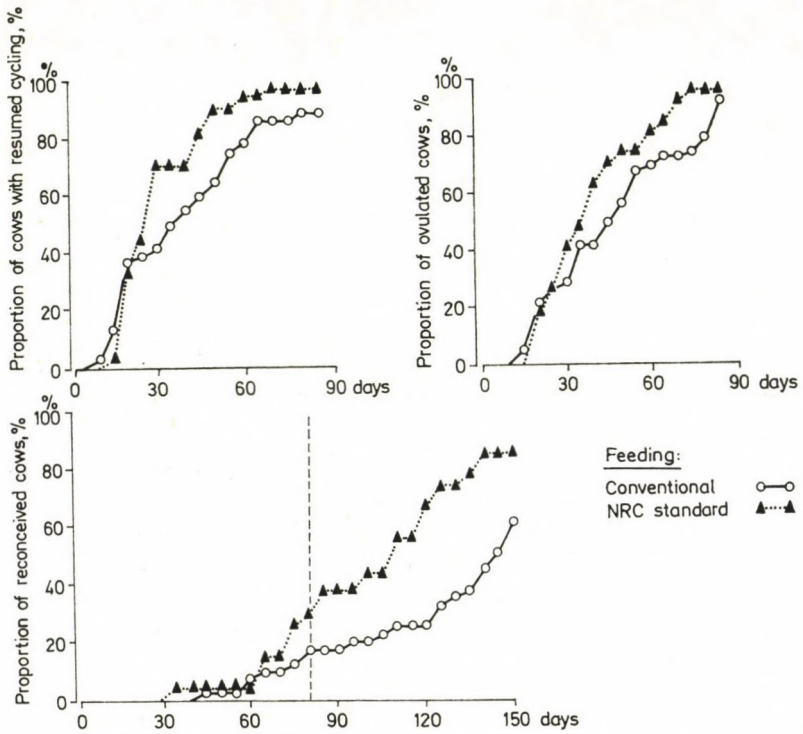


Fig. 3. Time-course of changes in the proportion of cows resuming cyclic ovarian activity, and of ovulated and reconceived cows in the conventionally fed group and in that fed according to the NRC requirements

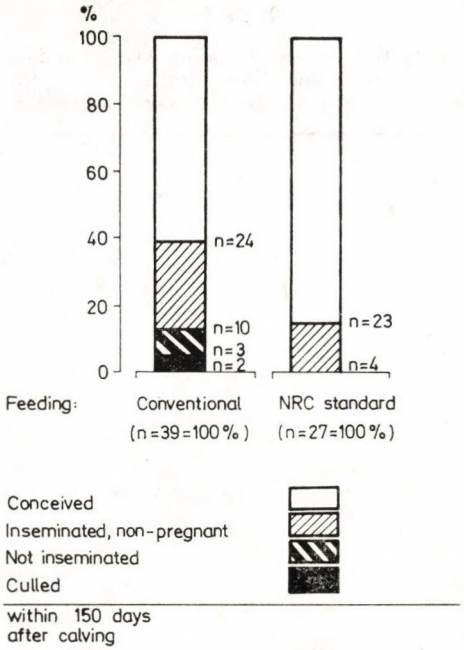


Fig. 4. Reproductive status of cows of the two feeding groups on postpartum day 150

Only 26 cows (39.4%) had normal ovarian function. Twenty-seven cows showed ovarian dysfuction once, 12 animals twice on two successive occasions, and one cow three times. Though no significant differences were found, in Group NRC the proportion of cows with delayed resumption of ovarian cyclicity (cycling re-established after day 35) was lower and cessation of cycling was not observed at all. The incidence of cystic follicular degeneration was about the same in the two groups (Fig. 2). In the proportion of cows with resumed cycling and of ovulated cows there were negligible, whereas in that of conceived cows marked, differences (Fig. 3). There were differences between the two groups in favour of Group NRC in reproduction status on postpartum day 150 (Fig. 4) and in the various reproductive indices (Table IV), too.

In Group C there was a strong positive correlation between milk production between lactation day 30 and 60 and the duration of postpartal acyclia. In Group NRC this correlation was much weaker (Table V).

Discussion

Multiparous cows of higher than average milk production and kept on two different feeding regimes were used in the present experiment. Comparison of the clinical findings with the progesterone profiles enabled us to monitor the re-establishment of cyclic ovarian function (Huszenicza et al., 1987).

Table V

Correlation between milk production between lactation days 30 and 60 and certain reproductive indices in conventionally fed cows and those fed according to the NRC requirements (as determined by a quadratic polynomic regression method)

	Feeding
	Conventional
Milk production between days 30 and 60	$Y = 98.79–2.394\ X + 5.649 \times 10^{-2} \times X^2$
– time of first postpartum insemination	$R = 0.105 \quad n = 34 \quad F = 0.1731$
Milk production between days 30 and 60 – time of conception	$Y = 316.2–19.61\ X + 4.285 \times 10^{-1} \times X^2$
	$R = 0.437 \quad n = 24 \quad F = 2.478$
Milk production between days 30 and 60 – duration of postpartal acyclia	$Y = 88.33–7.259\ X + 1.887 \times 10^{-1} \times X^2$
	$R = 0.582^{***} \quad n = 39 \quad F = 9.220$
	Feeding
	According to the NRC requirements
Milk production between days 30 and 60	$Y = 144.6–6.560\ X + 1.255 \times 10^{-1} \times X^2$
– time of first postpartum insemination	$R = 0.126 \quad n = 27 \quad F = 0.1942$
Milk production between days 30 and 60 – time of conception	$Y = 114.9–2.633\ X + 6.525 \times 10^{-2} \times X^2$
	$R = 0.223 \quad n = 23 \quad F = 0.5233$
Milk production between days 30 and 60 – duration of postpartal acyclia	$Y = -33.10 + 3.128\ X + 3.314 \times 10^{-2} \times X^2$
	$R = 0.361 \quad n = 27 \quad F = 1.798$

Remarks: [1,2] Based on data of cows inseminated[1] and conceived[2] within 150 days;
***: $P < 0.001$

The feeding of the two groups differed primarily during the dry period and in the early stage of lactation. In the dry period cows of Group C were fed energy levels substantially exceeding their actual requirement, whereas cows of Group NRC were supplied with energy levels close to the actual demand. During the first weeks of lactation the ration of Group C and Group NRC cows met the energy requirement of about 30 and 35 kg milk production, respectively. In addition, in the dry period cows of Group C were overfed with protein. The pathophysiological consequences of unjustifiably energy-rich feeding during the dry period are well known (Morrow, 1976). While in Group C the lowest-yielding cows, being immediately before drying-off, still received a markedly energy-deficient feed, cows of Group NRC were already able to build up some energy reserve.

Under the present experimental conditions, mostly identical with large-scale farm conditions prevailing in Hungary, presumably not even the feeding regime corresponding to the NRC requirements (1978) represented an optimum energy and protein supply for all cows used. This may have been due to the larger than desirable group size and the considerable individual variations of the lactation curves.

Differences in the incidence of involutional complications and course of uterine changes in favour of Group NRC are, in all probability, due to metabolic differences between the two groups. Several authors have observed that subclinical metabolic disorders (fat cow syndrome, ketosis) predispose cows to involutional complications (Morrow, 1976; Haraszti et al., 1982).

In ovarian function noteworthy but not sharp differences were observed between the two groups. In Group C ovarian disorders with energy balance disturbances as the underlying cause (delayed re-establishment of cyclic ovarian activity, irregular cycles, cessation of cycling) were more frequent (Huszenicza et al., 1988). Numerous observations have proved a relationship between the resumption of cycling and the energy balance and its disorders (Butler et al., 1981; Reid et al., 1983; Watson and Harwood, 1984; Haraszti et al., 1985). Therefore, we explain the difference found in the incidence of ovarian disorders in favour of Group NRC by the dissimilar frequency of subclinical metabolic disturbances.

Typically, in Group C there was a rather close correlation between the daily milk production between lactation days 30 and 60 and the duration of postpartal acyclia. A similar phenomenon has already been reported (Huszenicza et al., 1987). In Group NRC, however, only a moderate correlation was found between the two indices. This is consistent with results of other authors (Bulman and Lamming, 1978; Bulman and Wood, 1980; Pirchner et al., 1983) rather than with our previous findings. All these results suggest that if the energy supply is well balanced and close to the actual demand, the limiting effect of ovarian function on milk production, a property considered typical of cattle (Lamming, 1978), hardly manifests itself or is completely absent.

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OVARIAN ACTIVITY AFTER THE FIRST CALVING IN COWS REARED UNDER DIFFERENT FARM CONDITIONS

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Ovarian activity of primiparous Holstein-Friesian crossbred cows reared under different farm conditions was monitored by regular clinical examinations and by progesterone assay of milk samples collected after calving, at 3- to 4-day intervals, over a period of about 12 weeks. Of the ovarian disorders only prolonged postpartal acyclia proved to be rather common (55.8%). This primarily occurred in Group I (cows of poor body condition, small body size and fed rather poorly throughout) and Group II (small-sized but overfed cows initially kept on a poor, then, in the last trimester of pregnancy, on an unjustifiably rich feed). Ovarian activity and conception were the most favourable in Group III (well-developed cows of medium body condition, showing well-balanced development throughout). In Groups I and II there was a significant positive correlation between the daily milk production of cows at the peak of lactation and the duration of postpartal acyclia ($r = 0.791$; $P < 0.01$, and $r = 0.445$, $P < 0.1$ in Group I and Group II, respectively). No such correlation was demonstrable for Group III ($r = 0.197$, $P > 0.1$).

Keywords: Ovarian activity, primiparous cows, post partum, feeding

In primiparous dairy cows cyclic ovarian activity is usually resumed a few days later than after the second or third calving (Bulman and Wood, 1980; Claus et al., 1983; Huszenicza et al., 1987). This may be attributed to the fact that young cows have an extra nutrient requirement for growth, in addition to that required for maintenance and milk production (Balch, 1972). The complexity of this problem is, however, indicated by observations that the resumption of cyclic ovarian function could not always be appreciably influenced by varying dietary energy levels fed in the peripartal period (Carstairs et al., 1980).

Conception difficulties of primiparous heifers are rather common on the large-scale farms of Hungary. This prompted us to investigate this problem in detail. The purpose of the present study was to monitor ovarian function in primiparous heifers of medium or higher milk production and reared under different farm conditions, with different intensity.

Materials and methods

The study was conducted in three dairy herds comprising 600–800 Holstein-Friesian (HF) × Hungarian Fleckvieh (HFl) crossbred 3/4 HF × 1/4 HFl) primiparous cows each. The cows were kept under loose housing conditions. Heifers derived from dams of medium or higher than medium production calved between 25 May and 30 June.

Corresponding to the general practice, up to 60–90 days of age the heifers were fed on different milk replacers supplemented with concentrates and for-

Table I

Feeding of cows at farm A (Group I)

		Corn silage	Crushed corn grain	Meadow hay	Green lucerne, chopped	Dairy diet[2]	Preparatory cow diet[2]
		Daily ration, kg/cow					
Dairy cow groups	30 kg	10	3.7	4	15	3.2	—
	25 kg	10	2.9	4	15	2.3	—
	20 kg	10	2.2	4	15	1.3	—
	15 kg	10	0.8	4	15	0.2	—
First 6 weeks of the dry period		11	—	4	15	—	—
Last 2 weeks of the dry period		11	—	4	15	—	2.5
		Contents					
Dry matter	%	37.1	72.1	88.1	22.0	91.2	90.0
Crude protein	%	3.7	4.5	13.2	4.6	16.5	17.0
Crude fat	%	0.8	2.2	1.8	0.9	2.7	2.9
Crude fibre	%	9.6	2.8	25.6	5.4	3.6	7.4
N-free extract	%	21.1	58.5	40.4	8.2	61.6	59.6

		Nutrient contents of the ration					
		Requirements[3]		Actual contents		Difference	
		NE MJ[1]	Crude protein g	NE MJ[1]	Crude protein g	NE %[1]	Crude protein %
Dairy cow groups	30 kg	130.1	3066	105.4	2280	−18.9	−25.6
	25 kg	114.6	2631	94.5	2096	−17.5	−20.3
	20 kg	99.1	2196	83.5	1900	−15.8	−13.5
	15 kg	83.6	1761	67.8	1656	−18.9	−5.9
First 6 weeks of the dry period		50.7	868	64.4	1625	+27.0	+87.2
Last 2 weeks of the dry period		62.3	1178	79.1	2052	+27.1	+74.2

[1] Composition: 25% dairy cow diet (conc.), 72% corn, 3% fodder yeast;
[2] Composition: 97% bran, 3% fodder yeast;
[3] based on the NRC requirements (1978)

Table II

Feeding of cows at farm B (Group II)

		Corn silage	Lucerne hay	Dairy cow diet[2]	Grass, freshly cut
		Daily ration, kg/cow			
Dairy cow groups	30 kg	12	4	8.0	12
	25 kg	11	4	5.5	12
	20 kg	11	4	2.9	12
	15 kg	11	4	1.4	12
First 6 weeks of the dry period		15	4	1.5	12
Last 2 weeks of the dry period		12	4	1.9	12
		Contents			
Dry matter	%	30.0	88.2	88.7	36.0
Crude protein	%	2.6	16.5	22.0	4.3
Crude fat	%	0.5	1.4	1.8	1.5
Crude fibre	%	8.4	21.7	7.7	10.5
N-free extract	%	17.1	40.8	51.4	17.2

		Nutrient contents of the ration					
		Requirement[2]		Actual content		Difference	
		NE MJ[1]	Crude protein g	NE MJ[1]	Crude protein g	NE_1 %	Crude protein %
Dairy cow groups	30 kg	133.4	3102	115.4	3014	−13.4	−2.9
	25 kg	117.8	2667	97.8	2511	−17.0	−5.8
	20 kg	102.4	2232	81.5	2016	−20.3	−9.7
	15 kg	86.8	1797	72.1	1730	−16.9	−3.7
First 6 weeks of the dry period		55.7	946	80.7	1854	+44.9	+96.0
Last 2 weeks of the dry period		67.3	1256	77.2	1852	+14.8	+47.4

[1] Composition: 40.5% corn, 20% fodder wheat, 37% extracted sunflower groats, 2.5% complete premix, [2] based on the NRC requirements (1978)

ages of varying quantity and quality on all three farms. Subsequently the animals were kept on poor-quality, mostly sandy-soiled, pasture and outside the grazing season in half-open sheds with access to an open run. They were fed hay and corn silage. On farm C 1.5 kg corn-grits was given daily throughout the heifer-rearing period. The heifers were inseminated at 16 months old (farms A and C) and 18 months old (farm B). At farm C the heifers were not inseminated before reaching 350 kg in body mass. The animals were transferred to the dairy farm in the 4th–5th month (farm B) and in the 8th month (farms A and C) of pregnancy; subsequently they were kept there under managemental and feeding conditions identical with those of the dry cows.

The dry and lactating cows were kept in groups of 80–100 each. After calving the cows, including our experimental animals, were assigned to the group of the highest yielding cows, from where they were moved to the group corresponding to their actual milk production level at milking-in the following month.

Feed (Tables I, II and III) was provided in two approximately equal portions daily. The concentrates and silage were given as a uniform mixture, and hay as well as green forages were offered separately. The energy and protein content of the daily ration was calculated from results of the actual chemical analysis, as described previously (Huszenicza et al., 1988). In evaluating the

Table III

Feeding of cows at farm C (Group III)

		Corn silage	Crushed corn-cob silage	Green lucerne, chopped[2]	Dairy cow diet[1]	Wheat bran	Meadow hay
		Daily ration, kg/cow					
Dairy cow groups	30 kg	14	4.0	12	4.6	—	4
	25 kg	13	3.7	12	3.0	—	4
	20 kg	10	2.6	15	2.1	—	4
	15 kg	10	1.5	15	1.7	—	4
First 6 weeks of the dry period		10	—	15	—	1	4
Last 2 weeks of the dry period		10	—	15	—	4	4
		Contents					
Dry matter	%	32.1	59.8	22.0	88.1	88.9	88.9
Crude protein	%	3.1	6.0	4.6	15.8	16.0	9.4
Crude fat	%	0.5	1.5	0.9	2.0	3.0	1.3
Crude fibre	%	8.5	4.3	5.4	3.5	7.5	30.5
N-free extract	%	18.3	42.8	8.2	57.5	57.4	41.5

		Nutrient contents of the ration					
		Requirement[3]		Actual content		Difference	
		NE MJ[1]	Crude protein g	NE_1 MJ	Crude protein g	NE_1 %	Crude protein g
Dairy cow groups	30 kg	133.4	3102	106.5	2328	−20.2	−24.9
	25 kg	117.8	2667	92.6	2027	−21.4	−24.0
	20 kg	102.4	2232	79.3	1864	−22.5	−16.5
	15 kg	86.8	1797	71.9	1735	−17.2	−3.4
First 6 weeks of the dry period		55.1	936	60.2	1536	+9.3	+64.1
Last 2 weeks of the dry period		66.7	1246	77.7	2018	+16.5	+62.0

[1] Composition: 25% dairy cow diet (conc.), 72% corn, 3% fodder yeast; [2]Content values on the basis of Hungarian standard no. 6860; [3] based on the NRC requirements (1978)

actual energy and protein supply status we considered that the nutrient requirement of the still growing, primiparous cows being in their first lactation exceeded the maintenance requirement of mature cows of indentical body mass by about 20% (NRC, 1978). The probable feed intake of an average cow in the different production groups was calculated from the composition of the ration and from the cows' live body mass, by the so-called unite d'encombrement method (INRA, 1978).

	Age at insemination	Body mass after day 260 of gestation
Farm A (Group I)	<550 days	<500 kg
Farm B (Group II)	≥550 days	≥500 kg
Farm C (Group III)	<550 days	≥500 kg

Healthy heifers that conceived upon first insemination were weighed after day 260 of gestation. Cows meeting the above criteria, differing from farm to farm, were selected for the experiment.

The cows were weighed regularly during the rearing period. From the available farm records the results of earlier weighings (between 170–200, 270–300, and 370–400 days of age, at the time of conception, and between day 70–100 and 170–200 of pregnancy) were collected. The cows were weighed again between postpartum days 3–10 and 45–60.

The treatment of involution disorders and the applied reproduction technology were the same as described previously (Huszenicza et al., 1987). Over a period of 12 to 13 weeks after calving, at 3- to 4-day intervals milk samples were collected and assayed for progesterone after defatting, without extraction, with an enzyme immunoassay (Solti et al., 1986). Simultaneously, rectal and vaginal examinations were performed at 7- to 14-day intervals. Reproduction data were recorded up to postpartum day 150.

The re-establishment of cyclic ovarian activity was evaluated by comparing the results of the clinical examinations and the progesterone profile. The period between calving and the appearance of progesterone concentrations reaching or exceeding 1.6 nmol/l, regarded as the first sign of luteal activity, was considered the period of *postpartal acyclia*. Re-establishment of ovarian function was considered *physiological* if postpartal acyclia was shorter than 35 days and subsequently the CL phases showed cyclicity. If acyclia lasted longer than 35 days, *delayed resumption of ovarian cyclicity* was spoken of. The methods applied rendered possible the diagnosis of other ovarian dysfunctions (cycles of irregular length, cessation of cycling, follicular or lutein cysts, persistent CL). For details and methods of the statistical evaluation see an earlier paper (Huszenicza et al., 1987).

Data of cows with premature delivery, calving difficulties and twin pregnancy were excluded from the evaluation as well as those of animals showing metabolic disorders or pathognomonic signs of organic changes at the time of the examinations. Data of cows with lower than expected milk production (those producing less than 15 kg of 4% fat corrected milk (FCM) daily between postpartum day 30 and 60) were also excluded.

Results

The study comprised a total of 43 cows. Cows of Group I (n= 12) had small body size and were in poor body condition at the first calving (at 804 ± ±17 days old). The body mass gain of Group II cows (n = 15) was initially similar to that of cows in Group I; however, in the middle and last trimester of pregnancy a rapid improvement occurred in their body condition. At the first calving (at 878 ± 44 days old) the cows had small body size but good body condition. In the weeks after calving their body mass significantly ($P < 0.05$) decreased. Cows of Group III (n = 16) showed a well-balanced development throughout and by the time of the first calving (at 784 ± 27 days old)

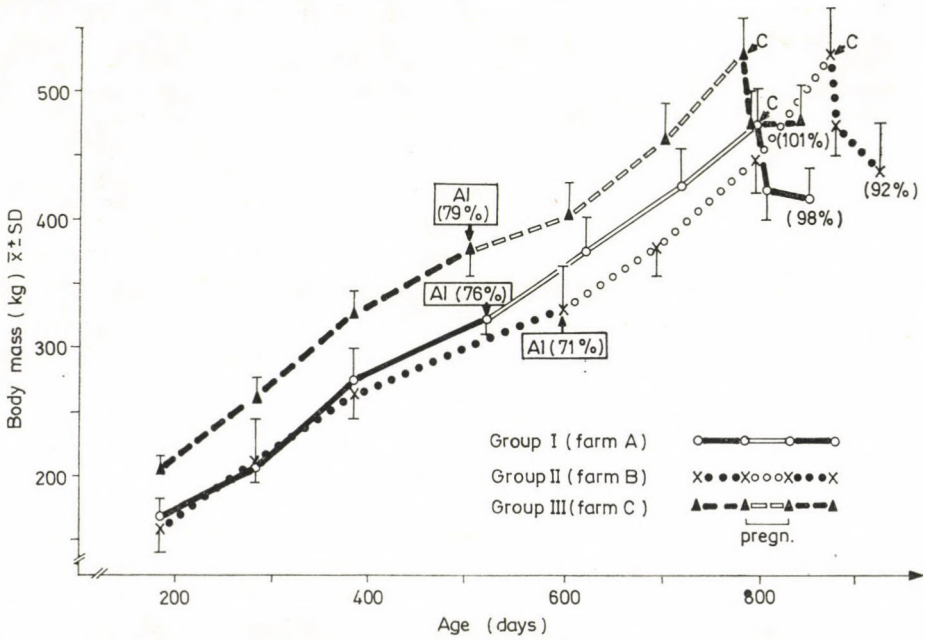


Fig. 1. Changes in the average body mass of the different groups from 160–180 days of age up to day 45–60 after the first calving. The body mass measured at conception and between day 45 and 60 post partum is expressed in per cent of the body mass found after calving. AI = time of artificial insemination and conception; C = time of calving

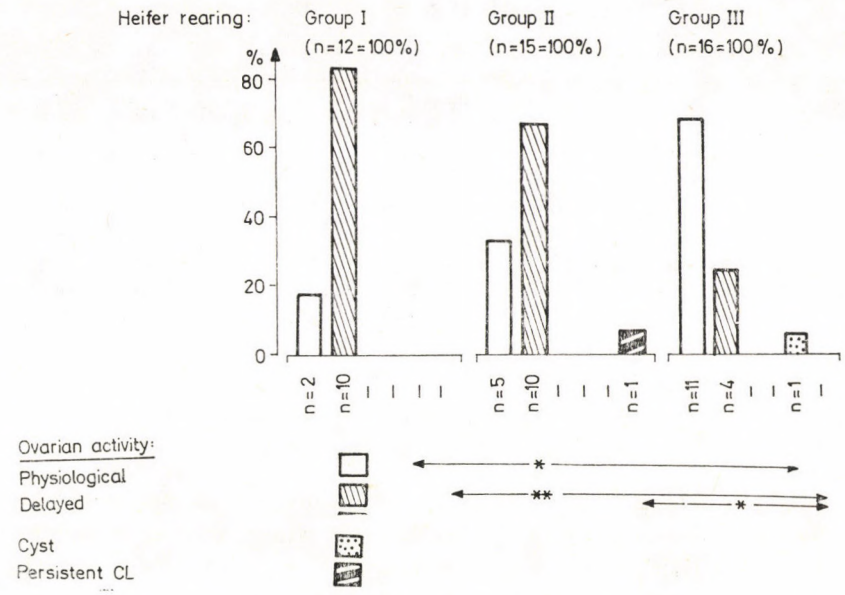


Fig. 2. Characteristics of ovarian function after the first calving in cows reared under different farm conditions

they had large body size and medium or somewhat better than medium body condition (Fig. 1).

Between day 30 and 60 of lactation Group I cows had the lowest daily milk production (19.8 $\pm$ 4.3 kg FCM). The milk production of Group II cows was slightly, while that of Group III cows significantly ($P < 0.05$) higher (21.9 $\pm$ 3.7 and 23.7 $\pm$ 4.6 kg FCM, respectively).

The incidence and duration of involutional complications were the most favourable in Group III, i. e. in well-developed cows of medium body condition (Table IV).

Resumption of cyclic ovarian function was physiological in 18 cows (41.9%). In 24 animals (55.8%) there was one, whereas in one heifer (2.3%) there were two successive ovarian dysfunctions. Of the ovarian disorders delayed resumption of ovarian cyclicity (beyond day 35) was predominant: it occurred in a total of 24 cases. Other disorders (cystic ovarian degeneration and persistent CL) occurred sporadically.

In Group I containing small-sized cows of poor body condition only two cows resumed cycling before day 35. In Group II the proportion of cows with physiological ovarian function was somewhat, while in Group III considerably, higher than in Group I (Fig. 2). This tendency was demonstrable in the proportion of cows with resumed cycling, and in that of the ovulated and

Table IV

Involution in heifers reared with different intensity

	Group I (n = 12 = 100%)		Group II (n = 15 = 100%)		Group III n = 16 = 100%)	
	n	%	n	%	n	%
Retained placenta	1	8.3	2	13.3	—	—
Involution						
— uncomplicated (no therapy required)	5	41.7	7	46.7	13	81.2
— complicated (therapy: <30 days)	3	26.0	2	13.3	3	18.8
— complicated (therapy: ≥30 days)	4	33.3	6	40.0	—	—

No significant difference was observed

conceived cows as well (Fig. 3). In Group II conception rate was unfavourable up to day 80; however, after day 90–100 it approximated that found for Group III. In Group I this index was considerably worse throughout than in Group I (Fig. 3). At postpartum day 150 the proportion of conceived cows

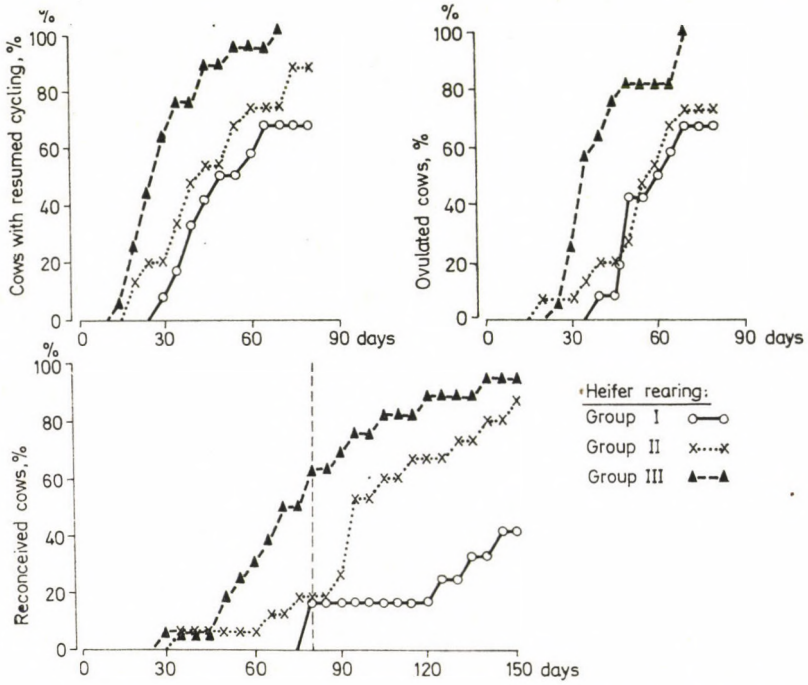


Fig. 3. Proportion of cows with resumed cycling, ovulated cows and conceived cows after the first calving

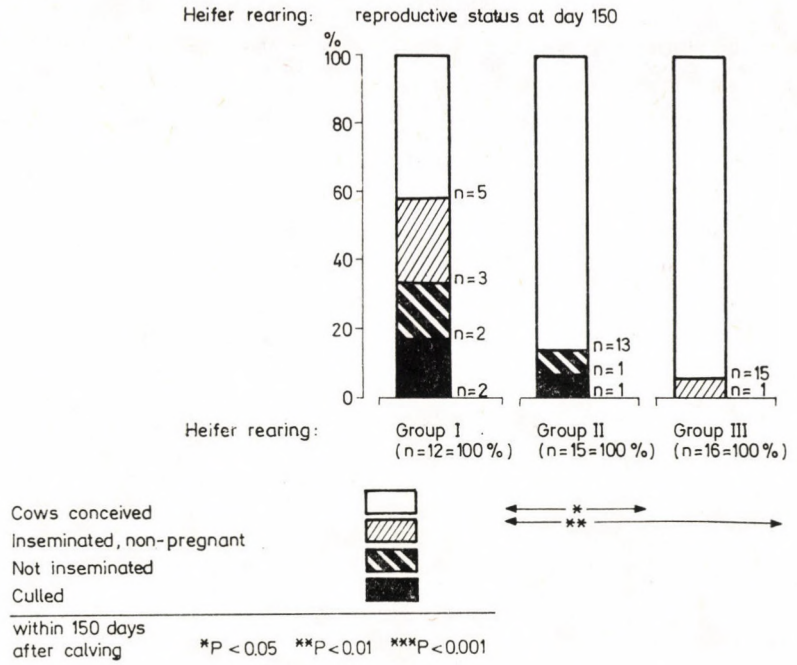


Fig. 4. Reproduction status of the experimental animals at day 150 after the first calving

was the lowest and that of open and culled cows the highest in Group I (Fig. 4). The intervals between calving and resumption of cycling, calving and first ovulation, calving and first insemination, and the calving-to-conception interval were the longest in Group I and the shortest in Group III (Table V).

Table V

Certain reproductive indices of heifers reared with different intensity

	Group I	Group II	Group III	P<		
	day, $\bar{x} \pm$ SD			I–II	I–III	II–III
Postpartum acyclia	57.2 ± 21.1	48.4 ± 21.0	38.8 ± 15.6		***	**
Start of the first CL phase $\geq$10 days	62.2 ± 18.4	58.9 ± 19.4	40.8 ± 14.9		**	**
First insemination[1]	82.1 ± 17.0	73.9 ± 38.1	67.1 ± 26.8			
Conception[2]	110.4 ± 27.4 (n = 5)	97.2 ± 27.8 (n = 13)	75.3 ± 28.0 (n = 15)		*	*

[1,2] data of animals inseminated[1] and those conceiving[2] within 150 days after calving;
* $P < 0.01$; *** $P < 0.001$; CL = corpus luteum

Table VI

Correlation of milk production between days 30 and 60 with certain reproductive indices as determined by linear regression from data obtained after the first calving for heifers reared with different intensity

	Group I	Group II	Group III
Milk production between days 30 and 60 –	Y=4.202+4.434·X	Y=19.801+2.421·X	Y=102.813–1.509·X
– time of the first postpartum AI[1]	R=0.391 n=8	R=0.299 n=13	R=0.258 n=15
Milk production between days 30 and 60 –	Y=0.5077+6.377·X	Y=17.473+3.650·X	Y=42.065+1.263·X
– time of reconception[2]	R=0.694 n=5	R=0.558* n=13	R=0.279 n=15
Milk production between days 30 and 60 –	Y=20.21+3.914·X	Y=4.041+2.024·X	Y=46.058–0.6276·X
– duration of postpartum acyclia	R=0.791** n=12	R=0.455(*) n=15	R=0.197 n=16
Duration of postpartum acyclia –	Y=1.177+2.758·X	Y=17.973+1.786·X	Y=10.958+0.9481·X
– time of reconception[2]	R=0.851(*) n=5	R=0.877*** n=13	R=0.634* n=15

[1,2] data of animals inseminated[1] and those conceiving[2] within 150 days after calving;
*) $p < 0.10$; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

By linear regression in Groups I and II a characteristic correlation was demonstrable between daily milk production at the peak of lactation and some reproductive indices (duration of postpartal acyclia, time of conception). At the same time, in large-sized heifers calving in medium body condition the production and reproductive indices were practically independent. The interval between calving and resumption of ovarian cyclicity and the calving-to-conception interval were significantly correlated, though the degree of this correlation varied by group (Table VI).

Discussion

From the body mass gain data it appears that, as compared to the recommendations of the North American Holstein Breeders' Association (cit. Bozó et al., 1975), in the rearing period cows of Groups I and II were poorly developed, whereas those assigned to Group III were in medium or good body condition. Cows of Group I and Group III maintained their body condition up to calving. At the same time, cows of Group II were bred later and in the middle and last trimester of pregnancy they were fed abundantly to compensate for poor feeding in the rearing period. Since, however, in that period growth rate is already lesser, by the time of calving these cows had got in a better than

desirable body condition. With knowledge of the body mass, feeding and production data it can be stated that for about one month after calving (i. e. until all experimental cows were fed the ration of the highest yielding cows) energy-deficient status need not be reckoned with at a medium dry matter intake (except for a few cows with a daily milk yield reaching or exceeding 30 kg). The considerable decrease in body mass, observed in Group II after calving, can be explained by the fact that better than desirable body condition limits dry matter intake, a phenomenon that has also been observed by others (Bellows and Short, 1978).

The re-establishment of cyclic ovarian function and the incidence of ovarian disorders were as expected on the basis of data of the literature (Bulman and Wood, 1980; Claus et al., 1983) and our experience gained earlier (Huszenicza et al., 1987). Ovarian dysfunctions other than delayed resumption of cycling rarely occurred. At the same time, there were significant differences in postpartal ovarian activity between groups of primiparous heifers reared under different farm conditions. These differences exerted a significant influence on conception. Heifers that had received poor feeding in the rearing period (Group I) resumed cycling after a longer period of acyclia and conceived later than Group III cows whose development corresponded to the breed standard. The adverse consequences of deficient feeding could not be prevented by energy supplementation given in the second and third trimester of pregnancy (Group II). Differences due to rearing with different intensity were marked despite the approximately similar energy supply in the postpartum period. The present results support the findings of Ducker et al. (1985) who, in a controlled model experiment, observed delayed onset of oestrus, ovulation, and conception in heifers that had received energy-deficient feed before calving. In Group II cows that were in better than desirable body condition before calving and lost weight in the first few weeks of lactation, the resumption of ovarian cyclicity was almost as unfavourable as in Group I cows whose body condition was poor throughout. The adverse effect of deteriorating body condition on ovarian activity after the first calving was also reported by Ducker et al. (1985).

An appreciable correlation between milk production and parameters indicative of the resumption of cyclic ovarian function was demonstrable only in cows that had been poorly fed earlier (Groups I and II). This suggests that milk production exerts an influence on the resumption of ovarian cyclicity only if energy supply disturbances are present. If the energy supply and the cows' appetite are good, lactation *per se* has no suppressive effect on ovarian function in young animals either, as it is considered typical of the species (Lamming, 1978).

In summary: despite inaccuracies and errors stemming from the type of the present experiments conducted at three different farms, it can be estab-

lished that markedly deficient feeding of heifers in the rearing period delays resumption of cyclic ovarian function and conception after the first calving. These adverse consequences cannot be prevented by breeding the heifers later or by giving them energy supplementation during pregnancy.

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COMPARATIVE STUDIES ON THE ANTIBACTERIAL DEFENCE MECHANISM OF VOLATILE FATTY ACIDS IN FIVE ANIMAL SPECIES

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Volatile fatty acids (VFA) present in the large-intestinal contents of rabbits, swine, cattle, sheep and broiler chickens were assayed by gas chromatography, while the antibacterial action of VFA on *Escherichia coli* was studied in vitro.

In healthy specimens of the animal species tested, the mean VFA concentration was between 68 and 128 mmol/kg, and was made up of acetic acid, propionic acid and butyric acid. The pH of the large-intestinal contents was between 5.8 and 7.2.

At pH 6.0, an antibacterial effect on *E. coli* was demonstrable in the large intestine of all five species tested. This antibacterial action, exerted by non-dissociated molecules of VFA, disappeared when the pH of the samples was raised to 7.0. At pH 6.0 6% of VFA are present in the non-dissociated form, and at this pH an antibacterial effect was demonstrated. At pH 7.0, however, the proportion of non-dissociated VFA molecules drops below 1% and no antibacterial effect is demonstrable in these samples.

The antibacterial action of VFA was of similar intensity in the large-intestinal contents of all five species. This VFA-associated defence mechanism may contribute to the prevention of enteric infections caused by bacteria.

Keywords: Intestinal tract, volatile fatty acids, antibacterial defence mechanism

Macroorganisms possess different defence mechanisms against bacterial infections. The best known and most significant of these are phagocytosis and the various immunobiological processes. The antibacterial effect of volatile fatty acids (VFA) present in the intestine is one of the defence mechanisms. In the intestine this mechanism should prevent invasion by pathogenic bacteria; at the same time, it must facilitate bacterial activity necessary for normal intestinal functions and digestion. This peculiar dual task is performed by VFA formed in the intestinal tract. They exert their activity in places where, due to the slower peristalsis, intestinal contents are stagnant over a long period and, in the absence of a proper defence mechanism, pathogenic bacteria can multiply excessively.

The antibacterial action of VFA in the intestinal tract was first observed in experiments conducted on germ-free and gnotobiotic animals. The presence of VFA in the intestinal tract of mice increased their resistance to infections by *Salmonella typhimurium* and *Shigella flexneri* (Miller and Bohnhoff, 1962; Freter, 1962; Bohnhoff et al., 1964; Hentges, 1967). Subsequently it was found that these VFAs also hindered invasion of the murine intestinal tract by

Pseudomonas aeruginosa and *Escherichia coli* (Lewison, 1978; Rodney and Owens, 1979).

Of the farm animals, the rabbit was the one in which VFA-associated antibacterial action was first demonstrated. Investigating the pathomechanism of *E. coli* diarrhoea, a common condition in weaned rabbits, it was discovered that an impairment of the intestinal defence mechanism had led to multiplication of *E. coli* (Prohászka, 1980; Prohászka and Baron, 1981). The VFA-associated defence mechanism has also been incriminated in the pathomechanism of *Treponema hyodysenteriae* infection of swine. When fed on diets producing a base load, the antibacterial effect of VFA diminished in the pigs' large intestine, resulting in multiplication of treponemes and onset of the clinical signs (Prohászka and Lukács, 1984).

Experiments on rabbits and pigs have revealed that in the large intestine the concentration and composition of VFA is controlled by a bioregulation which keeps the values within a range characteristic of the given species (Prohászka and Szemerédi, 1984; Prohászka, 1986). The above studies have shown that the decrease of intestinal antibacterial activity is due to an increased dissociation of VFA, which, in turn, depends on the pH of the environment. While at pH 6.0 there is a bactericidal effect in the large intestine, at pH 7.0 no antibacterial action could be detected whatsoever (Prohászka, 1986).

The results outlined above allow us to conclude that the antibacterial effect of VFA can have a role in the pathomechanism of enteric infections in different species of animals. In the present work, comparative studies on the antibacterial action of intestinal VFA carried out on samples from five species (rabbit, swine, sheep, cattle and chicken) are reported.

Materials and methods

Experiments with rabbits. Meat-type hybrid rabbits were used. Two different diets were used to monitor the effect exerted by the feed on the defence mechanism. One group (m = 10) received a granulated rabbit feed (crude protein: 17–18%, crude fibre: 11–12%) for 4 weeks. Rabbits of the other group (n = 10) were given forages (dried hay and alfalfa) instead of the rabbit diet. At the end of the 4-week trial, the rabbits were killed by bleeding and samples were taken from their caecal contents.

Experiments with pigs. Samples were collected on four large-scale pig farms. The pigs received a diet containing 15–17% crude protein, 4–5% crude fibre, and mineral, trace element and vitamin supplementation. Ten healthy pigs, weighing 30 to 80 kg, were selected for the experiments on each farm. After the pigs had been killed by bleeding, samples were collected from the distal portion of the large intestine.

Another group of pigs received a mixture of corn and barley (without any protein and premix supplementation) instead of the commercial fodder mix. This diet was fed to 6 pigs for 4 weeks. Pigs weighing 50 kg were killed by bleeding and samples were taken from the distal portion of the large intestine.

Samples from the intestinal contents of 10 wild hogs were examined to get an insight into the VFA-associated antibacterial effect functioning in the intestinal tract of wild animals. Adult wild hogs were bagged in December, and their large-intestinal contents were transported to the laboratory within 5 hours.

Experiments with sheep. Six adult sheep, weighing 40–50 kg, were kept on dried alfalfa hay and barley grains. Test samples were taken from the distal portion of the large intestine.

Experiments with cattle. Samples were taken from the distal portion of the large intestine of 10 healthy cattle slaughtered at an abattoir. The animals had been fed corn silage and dried hay.

Experiments with chickens. A total of 20 caecal contents samples, taken from 6 weeks old broiler chickens, were examined. The chickens received a starter, grower and finisher diet in accordance with the management technological requirements.

Assay of volatile fatty acids. The intestinal contents were diluted 1 : 10 with distilled water. By adding 50% H_2SO_4, the pH of the diluted samples was adjusted to 3. VFAs were determined by gas chromatography (Prohászka, 1980). VFA concentrations were expressed in mmol/kg, related to the original intestinal contents, whereas the proportion of acetic acid (C_2), propionic acid (C_3) and butyric acid (C_4) was given in %.

Study of the antibacterial effect. The intestinal contents were autoclaved at 120 °C for 20 min to destroy the original bacterial flora. Ten-gram quantities of the autoclaved samples were measured into two sterile glass containers. In one of them the pH was adjusted to 6.0, in the other to 7.0, by adding n HCl and n NaOH, respectively. To these samples an *E. coli* (O149 : K91, 88) suspension was added to reach a pre-incubation *E. coli* count of 10^4/g. The samples were incubated at 37 °C for 24 h. Subsequently, their *E. coli* counts were determined on 5% sheep blood agar, using the dilution procedure. A VFA-associated antibacterial effect was established when in samples of pH 6.0 *E. coli* counts did not increase, or even decreased, while in samples of pH 7.0 they exceeded 10^7/g.

The pH of the samples was measured with an electric pH meter (Radiometer PHM 84).

Results

Data on the VFA concentration and antibacterial action present in the intestinal contents of the animal species studied are shown in Table I. It is clear that the concentration of VFA in the intestinal tract varies with species:

Table I

pH values, volatile fatty acid (VFA) concentrations and VFA-associated antibacterial effect (at pH 6.0 and 7.0) in the large intestine of the animals species studied ($\bar{x} \pm SD$)

Animal species	Feed	n	pH	Volatile fatty acids, mmol/kg	C_2	C_3	C_4	Antibacterial effect at	
								pH 6.0	pH 7.0[3]
Rabbit	commercial diet	10	6.1 ± 0.3	72 ± 10	80	5	15	+[2]	—
Rabbit	hay	10	6.0 ± 0.2	68 ± 15	75	8	17	+	—
Pig	commercial diet	40	6.5 ± 0.7	116 ± 20	65	22	13	+	—
Pig	barley and corn	6	6.3 ± 0.3	108 ± 16	63	22	10	+	—
Wild hog	food[1]	10	6.6 ± 0.5	96 ± 35	64	27	9	+	—
Sheep	hay	6	6.7 ± 0.3	85 ± 11	65	26	9	+	—
Cattle	silage and hay	10	6.4 ± 0.4	110 ± 12	54	36	10	+	—
Chicken	commercial diet	20	6.7 ± 0.4	129 ± 22	54	31	15	+	—

[1] food found in the forest; [2] antibacterial effect present; [3] after adjustment by adding N HCl or N NaOH

the lowest mean value was obtained for the rabbit, the highest for the pig, chicken and cattle.

In samples of all five species acetic acid was the most abundant. As regards the propionic acid/butyric acid ratio, the rabbit differed from the other

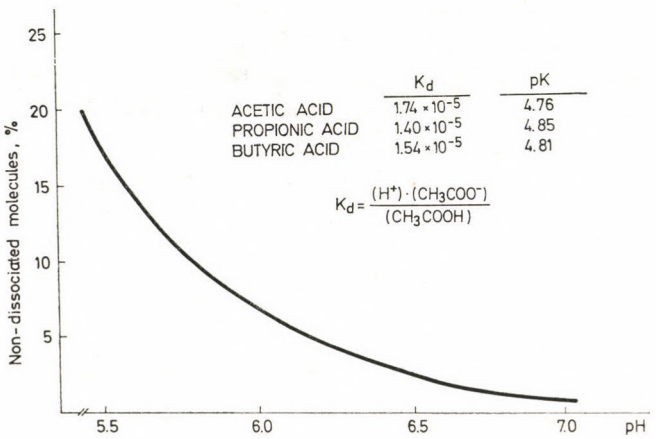


Fig. 1. K_d and pK of volatile fatty acids (VFA) present in the intestinal contents and the proportion of non-dissociated VFA molecules at pH values prevailing in the large intestine

four species tested. In the rabbit the proportion of butyric acid was higher than that of propionic acid, while in pigs, sheep, cattle and chickens the reverse was true. Practically no difference was found between samples taken from corn-fed pigs, wild hogs and rabbits fed on dried alfalfa hay and those originating from animals fed commercial diets. The mean pH of the samples was between 6.0 and 6.8 (extreme values: 5.8 and 7.2).

At a pH of 6.0, the large-intestinal contents of all five species showed an antibacterial effect. The *E. coli* counts of these samples decreased from 10^4/g to 10^2–10^3/g during an incubation period of 24 h. No substantial difference was found between samples from different species in the intensity of the antibacterial effect. When the pH of the samples was raised to 7.0, no antibacterial effect was demonstrable in any of the samples examined, in fact the *E. coli* counts rose to 10^7–10^8/g during incubation.

Discussion

We can conclude that the concentration of VFA in the large-intestinal contents of the five animal species was sufficient to permit demonstration of an antibacterial effect at pH 6.0. The composition of VFA was similar in the large-intestinal contents of all the five species tested: they mainly consisted of acetic acid, propionic acid and butyric acid. The rabbit differed from the other four species in that in its intestinal contents the concentration of butyric acid exceeded that of propionic acid. These results are consistent with earlier data of the literature (Henning and Hird, 1972; Imoto and Namioka, 1978).

The good agreement found between samples taken from rabbits and pigs, species fed different diets, proves that differences in feed composition do not alter the concentration and composition of VFA markedly. A similar conclusion was drawn by other investigators (Hoover and Heitman, 1972; Kennelly et al., 1981).

In the intestinal contents of the animal species studied, *E. coli* failed to multiply at pH 6.0; however, at pH 7.0 they multiplied without restraint. The study of Lewison (1978) showed that in the large-intestinal contents, within pH ranges of 6.0 and 7.0, the pH-dependent antibacterial effect is exerted by non-dissociated VFA molecules. The dissociation constants and pK values of acetic acid, propionic acid and butyric acid are shown in Fig. 1. The pK values of the three VFAs differ only slightly; thus, based on the equation, their dissociation status can be illustrated by a curve. This shows that at pH 6.0 6% of the VFA is in non-dissociated form. At the VFA concentrations present in the large intestine, this proportion is sufficient for an antibacterial effect. However, at pH 7.0 the proportion of non-dissociated VFA molecules drops below 1%, a proportion insufficient for an antibacterial effect.

The pH values of the large-intestinal contents collected from the five species of animals were between 5.8 and 7.2. Within this range a physiological fluctuation may occur. The pH of the large-intestinal contents is influenced by the quality of the diet fed, a fact that has been substantiated in feeding trials conducted with pigs. The large-intestinal contents of pigs fed corn silage (CCM) had a lower pH value; as a consequence, there was a stronger antibacterial effect than in the group fed commercial diets (Prohászka and Lukács, 1984).

During earlier studies we saw that at VFA concentrations prevailing in the large-intestinal contents an antibacterial effect was detectable up to pH 6.6. Beyond that value the antibacterial effect diminished in intensity; above pH 6.8 it was completely absent (Prohászka, 1986).

Detailed studies on the VFA-associated antibacterial effect in the intestinal tract have been performed only in the rabbit and pig, since in these species enteric diseases of bacterial aetiology are common. Few data are available on the role of the VFA-associated antibacterial effect in enteropathies of sheep, cattle and chickens. The data presented by us call attention to the fact that this defence mechanism may have a role also in the latter species of animals. However, further studies are needed to get a closer insight into this mechanism.

In the studies presented here, we used only *E. coli* for the demonstration of the antibacterial effect. However, in a previous paper we reported that VFA exerted a growth-inhibiting effect on other members of the Enterobacteriaceae family as well (Prohászka and Baron, 1982). Thus, the VFA-associated antibacterial effect functioning in the large intestine can be considered a nonspecific defence mechanism which should be reckoned with in enteric infections caused by bacteria.

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AN OUTBREAK OF *YERSINIA PSEUDOTUBERCULOSIS* INFECTION IN A BUSTARD *(OTIS TARDA)* FLOCK

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Deaths caused by a *Yersinia pseudotuberculosis* serotype I strain are reported from a bustard *(Otis tarda)* flock. The isolate was tested by experimental infection of mice, chickens and a turkey, and its antibiotic sensitivity was determined. It was revealed that the turkey can be a latent carrier of *Y. pseudotuberculosis.*

Keywords: *Yersinia pseudotuberculosis*, bustard *(Otis tarda)*, serotyping, antibiogram.

Yersinia pseudotuberculosis infection was diagnosed in connection with deaths in a bustard *(Otis tarda)* flock reared under semi-natural conditions in a reservation. The health state of our wild birds is poorly known.

Y. pseudotuberculosis infection has not been reported from bustards so far. A deeper knowledge of the diseases, primarily those of infectious nature, is required to ensure survival and breeding of this protected bird species. Therefore, in addition to the aetiological diagnosis, we deemed important to reveal the source of infection and possible modes of spread.

Materials and methods

The examined flock consisted of 45 bustards: 14 three to five years old captured and 31 one year old birds that had hatched in incubators. Out of them, five birds died in the spring of 1980 during a six-week period.

The birds were fed mainly vegetable feeds, occasionally white mice were also offered.

After the gross pathological examination of two carcases, haematoxylin-eosin stained sections were prepared from the liver and spleen. The bacteriological examination of the spleen and liver was carried out on 5% blood agar plates incubated at 37 °C, under aerobic conditions.

The isolates were identified by Gram staining as well as on the basis of their biochemical properties (oxidase, catalase, gelatinase, ornithine decarboxylase, urease, beta-galactosidase, and nitrate reduction tests). The tests were carried out as described by Cowan and Steel (1974). Glucose, arabinose, maltose, mannitol, salicin, rhamnose, melibiose, trehalose, lactose and sorbitol

fermentation as well as hydrogen sulfide and indole production were also determined. Motility was studied in Hitchens' semi-liquid medium at 22 and 37 °C.

Antimicrobial susceptibility was tested with Resistest disks (Phylaxia Veterinary Biologicals Co., Hungary).

To complete the bacteriological examination, the isolate was inoculated into two mice (body mass: 40 g), two chickens (14 days old, 200 g) and a turkey (14 days old, 250 g). The mice were inoculated intraperitoneally with 10^4 germs, the chickens subcutaneously with 10^5 germs, and the turkey was infected per os with 8 ml of a 24 hours old broth culture of the isolate daily, over a period of 8 days. Half of the bacterial suspension was poured on the feed and the other half into the drinking water.

The mice were kept under observation for 24 hours, one of the chickens for 14 days, the other one for 42 days, whereas the turkey for 49 days.

Gross pathological, histological and bacteriological examinations of the experimental animals were the same as those performed on the bustard carcases.

Results

Gross pathological examination of the bustard carcases revealed fibrinous inflammation of the airsacs and pericardium, enlarged liver, and hyperaemic splenomegaly. In the parenchyma of the spleen, well-visibly from the surface, there were greyish–white foci with indistinct margin and 1–3 mm in diameter. Similar foci were observed in the kidney and bone marrow. The foci contained necrotic, non-greasy substance and seemed to be of the same age on the basis of their size and nature. In structure the foci differed from those occurring in the early stage of generalized tuberculosis in that they did not have a connective tissue capsule.

Histopathological examination of the liver and spleen revealed necrosis and clumps of bacteria in the foci. Cellular elements characteristic of tuberculosis were not observed.

Bacteriological cultures of the liver and spleen yielded Gram-negative bacteria within 24 h. The bacteria showed no haemolysis, they were oxidase negative, catalase positive, beta-galactosidase positive, fermented urea, reduced nitrate, fermented glucose, arabinose, maltose, mannitol, salicin, rhamnose, melibiose as well as trehalose, and showed motility at 37 °C. The isolate did not ferment lactose and sorbitol, did not produce indole from tryptophan, did not produce hydrogen sulfide, and had no ornithine decarboxylase enzyme. On the basis of the above characteristics, the isolate was classified as *Yersinia pseudotuberculosis*.

The isolate was also identified by Professor W. Knapp (Nürnberg, Federal Republic of Germany) who classified it as serotype I according to its antigenic structure.

Table I

Antibiotic sensitivity of the isolated *Yersinia pseudotuberculosis* strain

			Diameter of the inhibition zone
Penicillin	R	Tetracycline	19 mm
Oxacillin	R	Nitrofurantoin	20 mm
Methicillin	R	Chloramphenicol	27 mm
Erythromycin	R	Streptomycin	25 mm
Oleandomycin	R	Gentamycin	29 mm
Cephalosporin	R	Oxytetracycline	23 mm
Spiramycin	R	Carbenicillin	28 mm
Pristinamycin	R	Kanamycin	26 mm
Vancomycin	R	Neomycin	26 mm
Lincomycin	R	Paromomycin	26 mm
Nystatin	R	Nalidixic acid	27 mm
Superseptyl	R		

R = Resistant

Mice inoculated with the isolate intraperitoneally died within 24 h. The bacteria were reisolated from the swollen spleen and liver.

The chickens were killed by bleeding 14 and 42 days after inoculation. Neither gross nor microscopic changes were observed in their organs and attempts at reisolating *Y. pseudotuberculosis* from the liver and spleen failed.

The apparently healthy turkey was exsanguinated 49 days after infection. Histological examination revealed diffuse pathological fatty degeneration in the liver, focal proliferation of reticulocytes in the red pulp, and lymphoid hyperlasia in the Malpighian corpuscles, of the spleen. *Y. pseudotuberculosis* was reisolated from the spleen and liver.

The antibiogram of the isolate and diameters of the inhibition zones are presented in Table I.

Discussion

The outbreak observed among bustards was caused by serotype I *Y. pseudotuberculosis*. This serotype is the commonest in Europe. It has not been reported from Hungary before.

The experimental infection of turkeys deserves attention. The susceptibility of turkeys to *Y. pseudotuberculosis* was described by Carter (1973). The latency period of the infection in turkeys is, however, unknown. Results of the histopathological and bacteriological examinations have confirmed Carter's findings and revealed that the turkey can be an asymptomatic carrier of this bacterium. Under certain conditions this may lead to *Y. pseudotuberculosis* outbreaks in turkey flocks (Thal, 1978). Stress conditions or differences in virulence may play a role in the manifestation of the disease.

Attempts at revealing the source of infection have failed. Latent infection of older bustards or the role of field mice and voles, however, could not be ruled out.

For the successful treatment of infected birds in zoos and reservations the antibiotic sensitivity of *Y. pseudotuberculosis* had to be determined. The sensitivity of bustards to antibiotics, however, should be elucidated before any antibiotic can be used in the practice.

Acknowledgement

The authors thank Professor W. Knapp, Head of *Yersinia* Reference Centre, Nürnberg, FRG, for his valuable help in serotyping the isolate.

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DIAGNOSTIC VALUE OF MAMMALIAN, AVIAN AND JOHNIN PPD TUBERCULINS IN CATTLE HERDS INFECTED BY *MYCOBACTERIUM PARATUBERCULOSIS*

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Results obtained on the diagnostic value of "paratuberculin PPD" serving for the diagnosis of paratuberculosis are reported.

Paratuberculin was applied in two different concentrations (0.86 mg/ml and 0.50 mg/ml tuberculoprotein), in a volume of 0.1 ml, and its effect was compared with that of mammalian and avian tuberculin.

The allergens were tested on three groups of animals by the intradermal test. The results were read in post-inoculation hour 72.

The first group consisted of 62 cows of a dairy herd free from parallergic signs (negative control group). In this group all allergens gave negative results.

The second group comprised 10 calves (positive control group) orally infected with cultures of *Mycobacterium paratuberculosis* twice. Tuberculin tests performed on days 92, 217 and 336 after the first infection indicated that, based upon the number and size of the reactions and the proportion of positive and doubtful reactions, the paratuberculin containing 0.50 mg/ml tuberculoprotein showed the highest sensitivity and specificity in diagnosing the infection.

The third group comprised a total of 1247 cattle of four farms, all infected by paratuberculosis (test group). The group contained both dairy and beef cattle, kept under loose housing conditions or free range management. In this group the paratuberculin containing 0.86 mg/ml tuberculoprotein and apparently also the avian tuberculin were of superior diagnostic value.

The results suggest that the paratuberculin containing 0.50 mg/ml tuberculoprotein is the allergen of choice for diagnosing the tuberculoid stage of paratuberculosis.

With knowledge of the pathogenesis of paratuberculosis, the parallel use of paratuberculin and avian tuberculin may be a valuable aid to the recognition and differential diagnosis of the early stage of paratuberculosis.

Keywords: *Mycobacterium paratuberculosis*, cattle, paratuberculosis, Johne's disease, paratuberculin, johnin PPD, tuberculins, diagnosis

In the pathogenesis of paratuberculosis (Johne's disease) two successive stages, i. e. the tuberculoid and the lepromatous stage, can be distinguished (Gilmour, 1985; Chiodini et al., 1984; Körmendy, 1985, 1986). In the preclinical or tuberculoid stage *Mycobacterium paratuberculosis* infection is characterized by the cell-mediated immune response, i. e. delayed-type allergic reaction (Körmendy et al., 1988). Animals in this immunobiological state can be identified by the lymphocyte-stimulation test and allergic diagnostic tests (Duncan et al., 1978). While the lymphocyte-stimulation test is unsuitable for herd testing, allergic tests using different tuberculins (mammalian and avian tuberculin, johnin) have found widespread application (Lepper and Corner, 1983).

Using strain no. 5889 Prague of *Mycobacterium paratuberculosis* a paratuberculin PPD was prepared and tested for sterility, innocuity, sensitizing properties, and potency. The paratuberculin PPD and bovine and avian PPDs were tested for their ability to identify infected animals in cattle herds affected by, and in those free from, paratuberculosis.

Materials and methods

Allergens. Mammalian tuberculin PPD, 50,000 IU/ml, Phylaxia, Budapest (designated M); 1 mg/ml tuberculoprotein (from strain AN 5).

Avian tuberculin PPD, 25,000 IU/ml, Phylaxia, Budapest (designated A); 0.5 mg/ml tuberculoprotein (from strain D 4).

Paratuberculin PPD; 0.86 mg/ml tuberculoprotein, Phylaxia, Budapest (designated P_1).

Paratuberculin PPD; 0.50 mg/ml tuberculoprotein; Phylaxia, Budapest (designated P_2).

To compare the tests, 0.1 ml of each allergen was inoculated into the skin of the neck of cattle in a herd infected by *M. paratuberculosis.* The tests were read in post-inoculation hour 72. The reaction was *positive* (+) if at the site of injection the skin had thickened by at least 3 mm as compared to its original thickness, or if the swelling was between 2 and 3 mm but was painful. The test was read as „*doubtful*" (±) if the swelling was between 2 and 3 mm in thickness and was not painful. If the skin swelled to some degree but the swelling did not reach 2 mm, the reaction was judged to be "*between negative and doubtful*" (∓). If the skin thickness measured on reading the test was practically identical with that assessed at the time of inoculation, the reaction was considered *negative* (—). For differential diagnostic reasons the size of reactions elicited by paratuberculin was assessed in the same way and at the same time as those induced by mammalian and avian tuberculin, though some authors recommended other reaction sizes and an earlier reading time (Chiodini et al., 1984).

Negative control group. The negative control group consisted of 62 Hungarian Fleckvieh cows of different age, kept in tying stalls at a large-scale farm free from *M. paratuberculosis* and *M. bovis* infection.

Positive control group. The positive control group was comprised of 10 bullcalves free from *M. bovis* infection. The calves were infected orally with 10^8 CFU of *M. paratuberculosis* strain 5889 twice, over five consecutive days each, at an interval of two weeks. The first infection took place when the calves were 2–3 weeks old. On day 92, 217 and 336 after the second infection the animals were examined by the intradermal test. The results are summarized in Table I.

Table I

Results of the allergic tests (thickness of the skin fold in mm)

Animal no.		Mammalian tuberculin on PI day			Avian tuberculin on PI day		
		92 I	217 II	336 III	92 I	217 II	336 III
1		7	—	—	13	—	—
2		6	1	2	12.5	5.2	2
3		2.5	—	2	6.5	—	2.5
4		6.5	2	2	15	5	1
5		5	1	3	13	2.1	4
6		3	1.5	—	10	—	1.5
7		—	2	2	—	5	5
8		4	1.5	2	11	7.1	4
9		2	1.5	2	7.5	0.5	2
10		5	4.1	3	7	8	—
Total:	+	7	1	2	9	5	3
	±	2	2	6	—	1	3
	∓	—	5	—	—	1	2
	—	1	2	2	1	3	2
	mm	40.0	14.6	18.0	95.5	32.9	21.5

Animal no.		P_1 tuberculin 0.86 mg/ml on PI day			P_2 tuberculin 0.5 mg/ml on PI day		
		92 I	217 II	336 III	92 I	217 II	336 III
1		5	—	—	8	—	—
2		9	1	2.5	11.5	4	4.5
3		3.5	2.9	3	7	—	3.5
4		6.5	6.1	2	14	4.2	3
5		8	1	1	13	7.1	3
6		2.5	—	—	6	—	—
7		—	2.8	2	—	—	4
8		5	3	3	8	4.5	4
9		5	1	—	7	1	—
10		6	5	3	10	9.2	3
Total:	+	8	3	3	9	5	7
	±	1	2	3	—	—	—
	∓	—	3	1	—	1	—
	—	1	2	3	1	4	3
	mm	50.5	22.8	16.5	84.5	30.0	25.0

Cattle of both the negative and the positive control group were kept in stanchion barns.

Test group. The test group included 1247 cattle kept at large-scale farms free from bovine tuberculosis and found to be infected, both histopathologically and bacteriologically, by *M. paratuberculosis*. The cattle were of the following breeds: Holstein-Friesian, Hungaro-Friesian, Limousin, Limousin × Hungar-

ian Fleckvieh, Hereford, Hereford × Hungarian Fleckvieh. The test group included both dairy and beef cattle, varying widely in age. The animals were kept either under loose housing or under free range management.

Results

The results are summarized in Tables I and II.

Negative control group. The intradermal tests performed on the 62 cows with mammalian (M) and avian (A) tuberculin and paratuberculins P_1 and P_2 were consistently negative. The herd in question had been negative by tests performed with the standard M tuberculin PPD for more than 10 years (not even doubtful reactions had occurred).

Positive control group. On day 92 and 217 after the first infection with *M. paratuberculosis*, the 10 bullcalves exhibited mycobacterium-induced seroconversion in intradermal tests with all the allergens used. Avian (A) tuber-

Table II

Comparison of allergic tests performed with mammalian tuberculin, avian tuberculin and paratuberculin PPD on 1247 cattle

	M+	M±	M∓	M−	Total
A +	32	27	19	59	137
A ±	6	11	12	50	79
A ∓	6	11	29	65	111
A −	5	12	45	858	920
Total:	49	61	105	1032	1247

	M+	M±	M∓	M−	Total
P_1 +	29	23	16	48	116
P_1 ±	5	12	13	39	69
P_1 ∓	5	10	19	60	94
P_1 −	10	16	57	885	968
Total:	49	61	105	1032	1247

	M+	M±	M∓	M−	Total
P_2 +	18	8	7	24	57
P_2 ±	14	10	4	28	56
P_2 ∓	1	16	22	44	83
P_2 −	16	27	72	936	1051
Total:	49	61	105	1032	1247

	A+	A±	A∓	A−	Total
P_1 +	65	17	18	16	116
P_1 ±	24	18	10	17	69
P_1 ∓	11	13	26	44	94
P_1 −	37	31	57	843	968
Total:	137	79	111	920	1247

	A+	A±	A∓	A−	Total
P_2 +	40	7	4	6	57
P_2 ±	24	15	9	8	56
P_2 ∓	22	14	20	27	83
P_2 −	51	43	78	879	1051
Total:	137	79	111	920	1247

	P_2+	P_2±	P_2∓	P_2−	Total
P_1 +	51	32	20	13	116
P_1 ±	2	18	24	25	69
P_1 ∓	1	3	25	65	94
P_1 −	3	3	14	948	968
Total:	57	56	83	1051	1247

A = avian tuberculin (PPD); M = mammalian tuberculin (PPD); P_1 = paratuberculin, concentrated (0.86 mg/ml); P_2 = paratuberculin, diluted (0.5 mg/ml); Reactions: + = positive; ± = doubtful; ∓ = between negative and doubtful; − = negative

culin and P_2 paratuberculin (containing 0.50 mg/ml tuberculoprotein) elicited the most pronounced skin swelling (Table I).

A comparison of intradermal tests with the four allergens on PI day 336 indicated that while the P_2 allergen elicited a positive response in 7 animals, with P_1 and A tuberculin only 3 animals each, and with M tuberculin only 2 animals were positive. The largest inflammatory reactions (25 mm) were induced by the P_2 allergen (Table I).

Test group. A comparison of allergic tests carried out on 1247 cattle (Table II) revealed that the M tuberculin, A tuberculin, P_1 and P_2 paratuberculins elicited a positive response in 49, 137, 116 and 57 animals, respectively.

The ratio of doubtful and positive reactions was determined for each allergen: with M tuberculin, A tuberculin, P_1 and P_2 paratuberculins this number was 1.25, 0.57, 0.59 and 0.94, respectively.

Discussion

In evaluating an allergen used in an allergic test, three factors have to be considered. The first is the immunobiological status of the animal to be tested, which depends on several factors, primarily on the stage of the disease process at the time of testing. The second factor is the specificity, and the third is the sensitivity of the allergen. Naturally, preparations intended for use in allergic tests should meet further requirements, such as sterility, lack of toxicity and immunogenicity, proper storability, adequate outer appearance, etc.

Theoretically, a good allergen should be strictly specific for the given bacterium species and should detect infection in any stage of the disease process so that it can demonstrate, by routine methods, the antigenic action exerted on the organism. Such an ideal allergen, however, does not exist. Therefore, the optimum tuberculoprotein concentration suitable for identifying, in routine tests, as many of the animals infected by *M. paratuberculosis* (in the tuberculoid stage of the disease) as possible, should be defined by purifying the allergens (PPD), adjusting their concentration, and by biological experiments performed on guinea pigs, cattle and sheep, on the basis of the doubtful/positive ratio and size of the inflammatory reactions at the different reading times.

The negative result of comparative tests carried out in the negative control group proved that our own paratuberculin preparations (P_1 and P_2) failed to exert an anaphylactogenic sensitizing effect and apparently did not cause any nonspecific reaction.

The results summarized in Table I demonstrate that in the tuberculoid or preclinical stage of paratuberculosis (Johne's disease) paratuberculins P_1 and P_2 are more specific and sensitive in identifying infected animals than

either mammalian or avian tuberculin. In the three experiments, the P_2 allergen identified 21, and the P_1 14 animals as positive, while 17 and 10 animals proved positive with A and M tuberculin, respectively (Table I). The superiority of P_1 and P_2 is indicated by results obtained for the test group (Table II) and the ratio of positive and doubtful reactions (1 : 0.94) which shows the optimal concentration of the allergen.

Considering only results obtained for the positive control group, the P_2 allergen seems the most efficient.

In the test group the paratuberculins were less effective in identifying infected animals than in the experimentally infected group: here avian tuberculin seemed to be more efficacious, since only 116 and 57 animals responded to P_1 and P_2, respectively. For this finding the following explanations may be considered:

(1) It cannot be ruled out with certainty that some or most animals of the test group had been infected, in addition to *M. paratuberculosis*, by other species of the Actinomycetales order: the higher positivity obtained with avian tuberculin than with the more specific paratuberculins might have been due to this fact. A theoretical explanation for this phenomenon was furnished by research into antigenic structure, which showed that a relatedness of varying degree existed in the complex antigenic structure of bacteria belonging to that group (Tuboly, 1965; Ratledge and Stanford, 1983).

(2) In the intradermal test each allergen was applied in a volume of 0.1 ml. Cattle kept in loose barns but even more those kept in free range management become irritable when approached by man. Thus, tests performed and read on animals restrained in stocks or treatment corridors may be a source of error. Presumably this is why P_1 paratuberculin appeared to be better than P_2. This allows us to draw further conclusions as to the practical applicability of the tests.

(3) As opposed to the test animals in free range management, cattle of the positive control group were housed in stanchion barns. In animals kept in tie-in stalls not only the intradermal tests are more reliable but, due to the lower risk of the otherwise common reinfections, the tuberculoid stage of paratuberculosis is prolonged. Animals of the positive control group kept in stanchion barns were shown to be in the tuberculoid stage of paratuberculosis. P_2 paratuberculin was found most suitable for identifying infected animals in that stage of the disease process (Table I).

(4) Cows of the test group ($n = 1247$) obviously were in different stages of paratuberculosis.

The above four paragraphs indicate the differences between results obtained for the positive control and the test group.

On the basis of the present experiments and results, paratuberculin PPD containing 0.5 mg/ml tuberculoprotein seems promising in identifying

animals affected by the tuberculoid stage of paratuberculosis. Presumably, this allergen will prove valuable in elucidating the cause of parallergic responses occurring when cattle herds are checked by tuberculin tests for freedom from bovine tuberculosis.

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A NEW, SIMPLE AEROSOL IMMUNIZATION TECHNIQUE AGAINST NEWCASTLE DISEASE

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A new aerosol immunization technique, involving the use of an aerosol generator, has been developed. Ninety to ninety-four per cent of the aerosol consists of particles ranging in size between 2.0 and 7.0 μm. The aerosol does not contain particles exceeding 7.0 μm in diameter. Another important element of the technique is the vaccine diluent: during the time of immunization (about 30 min) it substantially reduces losses by evaporation and prevents photo-oxidation of the virus. Using this technique, a La Sota virus dose of $10^{7.0}$–$10^{7.2}$ EID_{50}/m^3 is required to elicit a firm immune response at large-scale farms. In chickens younger than three weeks vaccination reactions may occur. Therefore, the technique is recommended for use in chickens older than three weeks.

The technique has been developed so that the aerosol generators are switched on and off outside the air-space treated. Operators shall enter the chickens house only after thorough ventilation.

By the technique substantial amounts (60–70%) of vaccine can be saved as compared to the methods used earlier. Immunization in a laying house containing 25,000 birds takes 40 minutes.

Keywords. Newcastle disease, La Sota strain, aerosol vaccination, particle size, vaccine diluent, immune response

Live virus vaccines can be used for mass immunization in large-scale flocks (Gough and Allan, 1973). Drinking water application of the lentogenic strains, however, may induce an insufficient level of protection (Balla et al., 1976). Therefore, the use of such strains by the aerosol route has become the centre of interest. Aerosol vaccination has been established as a quick and effective method (Allan and Borland, 1979). The technique should be simple, should induce a firm immune response in the birds, and should not give rise to adverse side effects.

The atomising performance and quality of aerosol generators developed so far (Atomist, Turbair, Pulsfog, DAG, etc.) have not rendered possible the generation of aerosols of sufficiently uniform distribution in large air-spaces. Two methods have been proposed and tried out for eliminating this practical problem. According to the first solution, the birds are immunized in a vaccination tunnel or crowded together in a narrow space for the time of vaccination. An alternative is when the operators hold the aerosol generator in their hands and direct the aerosol stream at the chickens. Both methods require that the operators stay in the air-space treated. If they do not wear close-fitting, ef-

fective gas masks, the operators may be exposed to risks of injuries to health such as conjuctivitis (Mayr, 1985). It should be mentioned that carrying aerosol generators for a period of several hours is a very exhausting work.

To the best of our knowledge, no aerosol vaccination technique is available that is applicable in large air-spaces (exceeding 500 m^3) without the operators staying there during the treatment. Therefore, our aim was to develop a technique which satisfies this demand.

Numerous papers have dealt with the role of the vaccine diluent in aerosol vaccination (Bondarenko et al., 1975; Ewert et al., 1977; Gough and Alexander, 1973; Tóth, 1959; Villegas and Kleven, 1976; Yadin and Orthel, 1978). Distilled water and protein solutions have proved the most effective. The aerosol generated from virus suspensions made with distilled water was tried out in such small air-spaces in which, as it can be implied from the experimental conditions, the virus may have got not only into the lower respiratory passages but, in the form of large droplets, also on the birds' conjunctiva and into their nostrils. These trials cannot be regarded as "pure" aerosol vaccinations and their results are not reproducible in large air-spaces. Protein solutions used as vaccine diluent, on the other hand, always pose the risk of causing allergic reactions.

The aim of the experiments was to develop an aerosol stabilizer which does not act as antigen but aids the generation of droplets that are uniformly distributed in large air-spaces and maintain their size for a given time.

Materials and methods

Aerosol generator. A MASTERDROP MD-2500 type aerosol generator, a modification of the EP-100 type equipment (Tamási et al., 1984), was used. The atomising performance of the generator is 2500 cm^3/h, and its weight is 7.8 kg.

Vaccine. NDV strain La Sota (Phylavac) was used. The vaccinal virus was titrated by inoculating embryonated hen's eggs incubated for 10 days. The titre was calculated according to Reed and Muench and expressed in $\log_{10}$ values. The calculated virus titres used in the experiments were $10^{6.5}$ and $10^{7.9}$ EID_{50} per cubic metre of air.

Determination of the size and number of aerosol particles. The number and distribution of particles in the air were determined by az AZ-5 type (UdSSR) instrument. Air samples were taken from areas of the air-space far from the aerosol stream. From the measured values we calculated the size distribution of particles in ranges of 0.5–1.0, 1.0–1.5, 1.5–2.0, 2.0–4.0, 4.0–7.0 and 7.0–10.0 μm. Mean particle diameter was determined by a laser particle counter (Chinoin Pharmaceutical and Chemical Works Ltd., Budapest).

Challenge infection. Vaccinations were performed under controlled conditons (temperature and relative humidity). The birds were challenged with 10^6 LD_{50} of virulent virus intratracheally.

Haemagglutination inhibition (HI) test. The HI test was performed in Takátsy's microtitrator trays. The geometric mean titres were expressed in $\log_2$ values.

Assay of antibiotics. Neomycin, oxytetracycline (OTC) and Tylan were assayed by a bactericidal test as described in Assoc. Off. Anal. Chem. (1970).

Vaccination at large-scale farms. The air-space of the chicken houses was calculated from the available data, or assessed, and was expressed in cubic metres of air. The performance of the MD-2500 type aerosol generator was determined in preliminary experiments on the basis of changes in the particle counts of the air. These results indicated that an aerosol generator kept in position was capable of causing a significant rise in droplet counts in an air-space of 500 m^3. Corresponding to the size of the air-space, 1 to 6 generators were distributed in the chicken house. Into the tank of each generator 50 g stabilizer was poured and the tank was filled up to the mark with tap water. Subsequently, we measured the resuspended vaccine into the tanks in doses enabling us to achieve the EID_{50}/m^3 values given above. The stabilizer was dissolved and the virus was resuspended by gently shaking the tanks. The doors of the chicken house were closed and ventilation was switched off. The generators were switched on from outside the air-space to be treated and were operated for 15 to 18 min. By the end of that time the tanks had been drained. Twelve to fifteen min after the generator had been switched off, ventilation was started again. The staff entered the chicken house only after 5–10 min of ventilation.

Results

The MD-2500 type aerosol generator was developed as follows.

The screened separator of the EP-100 type generator described in an earlier paper (Tamási et al., 1984) was replaced by a guide-railed separating tank. The internal diameter of the nozzle was increased. The shape, size and position of the nozzle were chosen experientially. They were adjusted until the largest droplets of the aerosol did not exceed 7 μm in diameter. Simultaneously, atomising performance and aerosol quality were adjusted to the desired values by optimum selection of the size, number, and position of the so-called guide-blades.

By these modifications the 0.84–0.96 dm^3/h atomising performance of the EP-100 type aerosol generator was increased to 2.5–3.0 dm^3/h without altering the electric motor. In the present experiments the new MD-2500

type (MASTERDROP) generator was used. Table I shows the comparison of the old and the new generator. In particle size distribution no considerable difference was demonstrable between the two types. Only in the number of the largest and smallest droplets was there an appreciable difference. Droplets larger than 7.0 μm were not measured for either generator. When the MD-2500 type generator was used, the highest particle count was $3–4 \times 10^5/dm^3$ in the various measurement ranges. The particle count was the same 20 and 160 cm above floor level. In non-stabilized aerosols generated from distilled water the particle counts of the air did not rise even in the droplet size class smaller than 0.5 μm: only relative humidity increased by a few per cent.

The effect of the photoprotective agent (PA) added to the stabilizer is demonstrated in Table II. At the time of vaccination the chicks had a low maternal antibody level (HI-GMT $\log_2$ 1.0). Blood samples were taken 15 days after vaccination. The birds were challenged on postvaccination day 24. If the virus concentration was higher than that shown in Table II ($10^{7.4}–10^{7.6}$

Table I

Distribution of particles according to size (in per cent of volume)

	Particle size (μm)	Time (min) elapsing after aerosolization					
		5	10	15	20	25	30
EP-100	0.5–1.0	3.90[a]	2.44	3.18	4.06	5.15	5.30
	1.0–1.5	3.30	3.34	4.95	4.62	5.19	6.69
	1.5–2.0	7.79	6.57	7.76	8.38	8.67	9.91
	2.0–4.0	66.49	75.05	72.67	73.46	73.63	71.43
	4.0–7.0	18.47	12.58	11.42	9.47	7.29	6.73
MD-2500	0.5–1.0	0.45	0.20	0.29	0.38	0.54	0.83
	1.0–1.5	1.66	1.37	1.40	1.78	2.22	2.86
	1.5–2.0	3.88	3.59	4.60	5.18	5.23	6.01
	2.0–4.0	59.67	62.73	69.98	68.65	71.77	72.74
	4.0–7.0	34.31	31.73	23.70	23.98	20.98	15.51

a: %

Table II

Effect of the photoprotective agent (PA) on the HI titres and results of challenge in chicks vaccinated at the age of 10 days ($10^{6.8}$ EID_{50}/m^3)

	With PA	Without PA
HI (GMT-$\log_2$)	4.90 (58[a])	2.97 (69)
Birds dying after challenge/total number of challenged birds	1/20	8/20

[a] number of chicks in the group

Table III

Vaccination reaction of aerosolized[a] chickens at the age of 21 days

Groups[b] treated	Respiratory reaction[c] (%)		Mortality[d] (%)	GMT-HI (log_2)
	Negative	Positive		
1 Neomycin[e] plus OTC	45	55	0	5.14
2 Tylan[e]	29	71	1.0	5.17
3 No antibiotics	26	74	2.5	5.27

[a] dose of La Sota: $10^{7.9}$ EID_{50}/m^3
[b] 200 chickens per group
[c] observed 6 days after vaccination
[d] occurring 5 to 9 days after vaccination
[e] 0.2 g/m^3 4 and 5 days after vaccination

Table IV

Mortality after challenge[a] in chickens vaccinated with NDV strain La Sota when 17 days old

Dose of La Sota EID_{50}/m^3 log_{10}	HI-GMT[b] log_2	Mortality (days after vaccination)			
		3	6	10	14
6.5	3.9	2/8[c]	0/8	0/8	0/8
6.9	7.1	1/8	0/8	0/8	0/8
7.1	7.1	2/8	0/8	0/8	0/8
Unvaccinated controls	0	8/8	8/8	8/8	8/8

[a] 10^6 LD_{50} intratracheally
[b] number of birds in each group: 60
[c] number of dead birds/total number challenged

EID_{50}/m^3), there was no difference according to whether or not the stabilizer contained PA.

A possible way of preventing vaccination reactions is shown in Table III. The chickens belonged to the so-called "hatchery waste" category and 15% of them died in spite of optimal management-technological conditions before reaching the age of 10 days. After deaths had ceased to occur, three groups, each containing 200 birds, were formed. The chickens were vaccinated at 21 days old: by that time the maternal antibodies had disappeared. The virus dose applied was larger ($10^{7.9}$ EID_{50}/m^3) than in the other experiments. On days 4 and 5 after aerosolization (on two occasions altogether) chickens of Group 1 received a mixture of neomycin and oxytetracycline, whereas those of Group 2 Tylan, both at a dose rate of 0.2 g/m^3. One to six h after treatment no antibiotics were detectable in the serum, lungs and liver of the birds killed by bleeding. From the lungs of the chickens dying during that period *Escherichia coli* was isolated.

Results of a challenge infection experiment are shown in Table IV. The majority of the chickens proved to be protected against challenge on postvaccination day 3. HI antibodies first appeared on days 6–7 after aerosol vaccination.

The results of immunization trials conducted in large-scale broiler flocks are given in Table V. A total of about 140,000 chickens were used. In experiments 1 and 2 dyspnoea occurred among the chickens, with a mortality rate of 1–2%. By the end of the fattening period the aerosolized chickens had higher mean HI titres in all experiments than the birds vaccinated in the drinking water.

Seroconversion of pullets vaccinated at 13 weeks old is shown in Table VI. Vaccination reactions were not observed. In the aerosolized chicken houses the HI titres were strikingly high, even as compared to the other experiments.

Table V

Vaccination of broiler chickens against Newcastle disease

Exp.	Age (day)	La Sota EID_{50} per m^3 log_{10}	Sampling after vacc. (day)	Neg. HI	HI (dilution of sera, 1:)												HI-GMT log_2
					2	4	8	16	32	64	128	256	512	1024	2048	4096	
1	19	7.0	33	—	—	4	23	23	25	11	5	5	—	—	—	—	4.5
2	20	DW[a]	15	—	—	3	2	7	11	8	4	2	1	2	—	—	5.4
	20	7.2	15	—	—	—	3	2	6	10	13	4	2	—	—	—	6.2
3	21	DW	28	2	5	5	5	6	6	1	—	—	—	—	—	—	3.0
	21	7.0	28	1	—	7	6	16	17	12	1	—	—	—	—	—	4.3
	21	7.0	28	1	1	8	10	13	16	8	2	—	—	—	—	—	4.1
4	21	DW	23	3	5	7	13	10	3	2	2	—	—	—	—	—	3.1
	21	6.9	23	1	2	5	7	17	21	12	—	—	—	—	—	—	4.3

Number of chickens in Exp. 1–4: 140,000
[a] in drinking water, $10^{6.5}$ to $10^{6.8}$ EID_{50} per chicken

Table VI

Vaccination of pullets at the age of 13 weeks

Route of admin.	Days after vacc.	Neg. sera (n)	HI titres (1:)												HI-GMT log_2
			2	4	8	16	32	64	128	256	512	1024	2048	4096	
In the drinking[a] water	0	2	4	8	8	5	2	1	—	—	—	—	—	—	2.6
	15	—	2	9	11	8	6	12	3	—	—	—	—	—	3.7
By aerosol[b]	0	—	—	12	10	3	5	—	—	—	—	—	—	—	3.0
	15	—	—	—	—	—	—	1	1	4	4	14	18	9	10.3

[a] $10^{6.6}$ EID_{50} per chicken
[b] $10^{7.1}$ EID_{50} per m^3
Group size: 5000 birds

Table VII

Vaccination of laying hens with Atomist and Masterdrop aerosol generators

Route of admin.	Neg. sera (n)	HI titres (1:)												GMT-HI $\log_2$
		2	4	8	16	32	64	128	256	512	1024	2048	4096	
Initial value	1	1	4	—	2	4	11	7	6	—	—	—	—	5.55
Atomist[a]	—	—	—	—	—	1	3	5	5	4	5	2	2	8.51
Masterdrop[b]	—	—	—	—	—	—	4	6	7	10	6	3	—	8.47

[a] $10^{6.5}$ EID_{50} per bird, n: 16,000
[b] $10^{7.0}$ EID_{50} per bird, n: 42,000

Table VIII

HI titres of aerosolized laying hens at the age of 31 weeks

Samplings after the aerosol (days)	HI (serum dilutions, 1:)										GMT-HI $\log_2$
	2	4	8	16	32	64	128	256	512	1024	
35	—	—	—	2	8	11	22	26	9	2	7.21
105	—	1	11	12	11	24	14	16	11	3	6.21

Number of vaccinated birds: 56,000 (in two houses)
Dose of La Sota strain: $10^{7.1}$ EID_{50} per m^3

Table VII summarizes the results of immunization performed with the Atomist and the MASTERDROP apparatus. The initial HI titres shown in the table reflect the result of an immunization performed with an inactivated vaccine four weeks earlier. There was no difference in the degree of protection induced. In efficiency, however, there was a substantial difference between the two methods. Namely, with the Atomist two operators vaccinated 16,000 chickens in two work-days, whereas with the MASTERDROP one operator immunized as many as 42,000 chickens in 3 h.

Data on the aerosol vaccination of laying hens are given in Table VIII. Egg production did not decrease after aerosolization. Three and a half months after aerosol vaccination the mean HI titres of the flocks exceeded 1 : 64.

Discussion

The most important achievement of the present work has been the development of an aerosol vaccination technique which can be carried out without having to move the birds and so that the operators need not stay in the air-space treated. This method reduces the health risks associated with

aerosol immunization. The efficiency of the work and the working conditions have been improved.

By the recommended technique substantial amounts of vaccine can be saved as compared to any other vaccination procedure. E. g. by our aerosol technique only $10^{10.3}$–$10^{10.5}$ EID_{50}, whereas in drinking water application or using the Atomist apparatus $10^{10.9-11.1}$ EID_{50} virus was needed for a laying house of 25,000 birds. This corresponds to about 60–70% savings in vaccine.

As compared to the aerosol stabilizers used earlier, the stabilizer developed by us does not contain foreign substances; it consists of well-definable compounds and can be produced in standard quality. (The stabilizer is commercially available). In earlier studies no attention was paid to protecting the vaccinal virus against photo-oxidation. Our experiments have shown that protection of the virus is very important, particularly if, for some reason or other, the virus dose is lower than planned. This primarily occurs after inadequate storage and transportation (Allan and Dawson, 1973). Inactivated vaccines that have recently become widespread are neither free from problems: they considerably increase the costs of immunization and keeping. Inactivated vaccines usually confer a good acquired immunity and do not cause vaccination reactions. However, local reactions do occur after their use and the adjuvant is demonstrable from the organism for as long as 28 weeks after vaccination (Jarc and Kölbl, 1984).

Our results support earlier findings (Gough and Alexander, 1973) according to which resistance to Newcastle disease developed a few days after aerosol vaccination despite the fact that HI antibodies had not appeared by that time (Giambrone, 1985).

We have found that the immunization of even 19–20 days old birds poses some risks. The cause of vaccination reactions should not be looked for in the vaccination technique itself. E. g. Hentges et al. (1984) found that after drinking water application of NDV vaccines the rate of protein synthesis decreased in the muscles. Renaut and Zygraich (1979) induced a vaccination reaction with droplets as large as 40–60 μm. We agree with the conclusion drawn by Villegas and Kleven (1975) that it is not fully correct to evaluate the immunogenicity of the so-called "coarse spray" since droplets larger than 3 μm immunize by inhalation only if their size decreases below 3 μm as a result of evaporation. Clear interpretation of the results is further hindered by the fact that in poultry the basic data on aerosol particle sedimentation in the respiratory passages apply to the so-called "dry particles" and are only partially true for liquid droplets (Hayter and Besch, 1973).

Since in our opinion the vaccination reactions cannot be regarded as problems connected with the vaccine application technique, the present experiments have yielded an effective, simple aerosol vaccination technique that does not pose a risk to human health and elicits a firm immune response.

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EPIZOOTIOLOGICAL STUDIES ON PORCINE ATROPHIC RHINITIS XV. RESULTS OF LOCAL IMMUNIZATION WITH A LIVE *BORDETELLA BRONCHISEPTICA* STRAIN IN A LARGE-SCALE PIG HERD AFFECTED BY ATROPHIC RHINITIS

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A natural avirulent *Bordetella bronchiseptica* mutant was isolated from a swine herd free from the clinical signs and gross pathological lesions of atrophic rhinitis (AR). In experiments with mice, the mutant proved to be atoxic. The strain could be reisolated from the nasal mucosa of SPF piglets for 21 days and was apathogenic for swine.

The live strain was used in four schemes of immunization experiments in four large-scale swine herds severely affected by AR. The inoculum was 5×10^8 CFU per nostril, applied on the nasal mucosa. The pig herds were infected by both exotoxin-producing *B. bronchiseptica* and exotoxin-producing *Pasteurella multocida* strains. In scheme I, the piglets were vaccinated at birth, in scheme II at birth and at 15 days old, in scheme III at birth and at 30 days old, and in scheme IV at birth and at 60 days old. The vaccinated groups contained 120–1800 piglets in the various herds.

The bacteriological, immunological and gross pathological examinations performed in the four herds have led us to the following main conclusions:

Immunization induced, within three weeks, protective mucosal immunity which prevented virulent *B. bronchiseptica* strains from colonizing the nasal mucosa, and reduced the severity of infection by virulent *P. multocida*. In piglets immunized once the protection against *B. bronchiseptica* lasted at least 4–6 weeks, while in those vaccinated twice as long as 2 to 3 months.

In the nasal secretion of the immunized piglets higher titres of antibodies were found than in the controls. These antibodies reacted with both the *B. bronchiseptica* and the *P. multocida* antigens.

In agreement with results of the bacteriological and immunological studies, the gross pathological findings indicate that two immunizations with the *B. bronchiseptica* strain may reduce the severity of AR by 65–80% in pig herds affected by AR, particularly if the second vaccination takes place at 30 days old.

Keywords: Atrophic rhinitis, swine, epizoo tiology, *Bordetella bronchiseptica*, local immunization, avirulent strain

Atrophic rhinitis (AR) of swine is known to be caused by exotoxin-producing *Bordetella bronchiseptica* and *Pasteurella multocida* strains (Duncan et al., 1966; Harris and Switzer, 1968; De Jong et al., 1980; Miniats and Johnson, 1980; Pedersen and Barfod, 1982; Rutter, 1983; Shimizu et al., 1971). Due to the infectious nature of the disease, immunization with a killed vaccine is used as a means of control in affected swine herds (Éliás and Török, 1984;

Goodnow et al., 1979; Harris and Switzer, 1972; Keller and Lorentz, 1980; Koshimizu et al., 1973).

Little is known about the local immune response of the nasal mucosa after either parenteral or local antigen application. In the nasal secretion of piglets parenterally immunized with a killed *B. bronchiseptica* vaccine, agglutinating antibodies appear which eliminate the virulent *B. bronchiseptica* strain used for intranasal infection (Brandenburg, 1978). Other authors demonstrated, in the nasal secretion of piglets intranasally infected with a low-virulence *B. bronchiseptica* strain, antibodies which substantially reduced the germ counts of the virulent strain used for local infection (Harris and Switzer, 1969; Wisecarver and Goodnow, 1980).

The most promising result was achieved by Harris (1970) who, following local immunization of swine with an avirulent live *B. bronchiseptica* strain (strain D-1), observed a resistance starting from postvaccination day 25 and lasting 125 days. As a result, the virulent *B. bronchiseptica* strain used for infection was eliminated in 2 to 6 hours. Similar results were reported by Farrington (1974) who demonstrated immunity of the nasal mucosa from postvaccination day 14.

In SPF piglets locally immunized with an avirulent live *B. bronchiseptica* strain, on day 21 after vaccination we demonstrated mucosal immunity against challenge with 5×10^8 CFU of virulent *B. bronchiseptica*. In the same experiment, during determination of the immunoglobulin content of the nasal secretion by indirect immunofluorescence (IF), a cross-reaction was found between *B. bronchiseptica* and *P. multocida* strains (Éliás et al., 1985).

The purpose of the present work was to elucidate the potency of local immunity and to determine the optimum time of vaccination in large-scale pig herds severely affected by AR.

Materials and methods

Large-scale pig herds

Four large-scale pig herds (A, B, C and D) were chosen for the experiment. In these herds the clinical signs of acute AR seen in the 6–10 weeks old piglets (sneezing, nasal discharge, lachrymation, contamination around the eyes) and the gross lesions found in the nose of fattening pigs at slaughter indicated high prevalence of the disease. In these herds both the exotoxin-producing *B. bronchiseptica* and serogroup D *P. multocida* strains were isolated from the nose of the clinically affected pigs.

Selection of the vaccine strain

The vaccine strain used in the experiment was selected from strains isolated from the nasal mucosa of pigs for slaughter in herds free from the clinical

signs and gross lesions of AR. The biological properties of the strains were determined by methods published by us earlier (Éliás et al., 1982).

The *capsular antigens* were obtained from an antigen suspension (10^{12} CFU/ml) washed down from blood agar plates incubated for 18 h. The suspension was kept in waterbath at 56 °C for 30 min, centrifuged at 5000 rpm for 30 min, and the supernatant was used for capsular antigen determination by agar-gel precipitation. Antisera were raised in rabbits against *B. bronchiseptica* (22067 and 899) and *B. parapertussis* (504) reference strains. The sera were absorbed with *B. bronchiseptica* (214, 22067, 899), *B. pertussis* (5373), and *B. parapertussis* (504) strains.

Haemagglutination was determined in a bacterial culture incubated for 18 h, containing 10^{10} colony-forming units (CFU) per ml. A $\log_2$ dilution series (up to 1 : 256) was made from the bacterial culture with 1 ml saline in agglutination tubes, and 0.05 ml volumes of 2% calf, horse and sheep red blood cell (RBC) suspensions, washed several times, were added to the dilutions. The mixture was shaken vigorously, incubated at 37 °C for 1 h, and refrigerated overnight at 4 °C. The test was scored as follows: $+++ = 100\%$ agglutination; $++ = > 50\%$ agglutination; $+ = < 50\%$ agglutination.

Determination of virulence. A $\log_{10}$ dilution series of a broth culture incubated for 18 h, containing 10^9 CFU per ml, was used. Mice weighing 12 g, anaesthetized with ether, were inoculated intracerebrally with 0.05 ml of the dilutions, those weighing 16 g received 0.1 ml intratracheally, and other mice, also weighing 16 g, 0.5 ml volumes intraperitoneally. Each group consisted of four mice. The period of observation was 7 days.

Albino mice weighing 20 g were used for the determination of *exotoxin production.* The mice were inoculated into the tail vein with 0.5 ml volumes of a $\log_2$ dilution series (1 : 2–1 : 16) of the 18-h broth culture of the strain. Four mice were inoculated with each dilution. Deaths were recorded over a period of six days, and on the 7th day we determined the relative spleen mass of mice killed in ether anaesthesia, in mg/10 g body mass.

The *pathogenicity and stability* of the strain were determined in SPF piglets. A three-week-old piglet was injected into each nostril with 0.5 ml of the 18-h broth culture containing 5×10^8 CFU. The 18-h broth culture of the strain reisolated on day 5 was inoculated into another piglet, then at five-day intervals it was passaged in a third and fourth piglet (infective dose: 5×10^8 CFU). Finally, the properties of the strain reisolated from the fourth piglet were determined.

The piglets were killed, necropsied and examined for gross and histopathological changes two months after infection.

Mode and schemes of vaccination

In the four pig herds the live vaccine strain was applied according to the following schemes:

Scheme I: vaccination at birth;
Scheme II: vaccination at birth and at 15 days old;
Scheme III: vaccination at birth and at 30 days old;
Scheme IV: vaccination at birth and at 60 days old.

In herd A, immunization schemes II and III were used on 160 piglets per group. In herd B, schemes III and IV were followed in groups comprising 120 piglets. In herd C, immunization schemes II, III and IV were applied; here the groups consisted of 1800 piglets. In herd D 160 piglets were vaccinated at birth (scheme I).

On a single vaccination 5×10^8 CFU of the lyophilized strain, dissolved in PBS, was injected into each nostril with a syringe. The piglets were kept head downwards during the immunization. The first vaccination usually took place within 6 hours after birth. During rearing, the piglets infected with the vaccine strain were kept isolated from other pigs in the unit but in the same air space.

Evaluation of the experiments

For *bacteriological examinations*, nasal swabs were taken from the same 20 pigs of all experimental groups and of the control group at 7, 21, 40, 90 and 120 days old. *B. bronchiseptica* strains were isolated on Bordet-Gengou and MacConkey media, and *P. multocida* strains on blood agar and on a selective medium containing 5% sheep blood (heart infusion agar, Difco; clindamycin phosphate 5 mg/l, gentamicin sulphate 0.75 mg/l, sodium tellurite 2.5 mg/l, amphotericin B (Sigma) 5 mg/l, bacitracin 15 mg/l). Six strains out of the *B. bronchiseptica* isolates were tested to determine whether or not the isolate was a mixture of several *B. bronchiseptica* strains of different properties. The properties of the *B. bronchiseptica* strains were determined as described above, whereas those of the *P. multocida* strains by the methods of Iordache et al. (1980) and de Jong et al. (1980).

Immunological studies were performed simultaneously with the bacteriological examinations. Piglets immunized according to scheme III (as a result of their favourable bacteriological findings) and the control animals were examined. On one occasion the immunoglobulin content of the nasal secretion of three pigs per group was determined by indirect IF. The results are given as extreme values. From 20-h *B. bronchiseptica* and *P. multocida* cultures of 10^8 CFU/ml germ count, smears were prepared and fixed with methanol for 20 min after drying. Log_2 dilutions (10–1280) of the nasal secretion were layered

onto the smears. The smears were dried for 20 min at room temperature and washed in PBS three times. The preparation was treated with 1 : 50 dilutions of rabbit anti-pig IgA and IgG for 20 min, then washed three times with PBS again. Finally the preparation was stained with FITC-labelled anti-rabbit Ig, washed three times with PBS, and examined in a Leitz-Orthoplan fluorescent microscope at a magnification of × 375. In a positive case the bacterial cells exhibited strong fluorescence. To obtain nasal secretion, 1 g of mucosa was removed from the turbinates of each animal, cut into small pieces, and soaked in 2 ml saline for 24 h at 4 °C. The secretion was centrifuged at 5000 rpm for 30 min, and the clear supernatant was used (for the sake of repeatability, the dilution rate was arbitrarily considered 1 : 5).

Gross pathological examinations were performed on 20 pigs of slaughter weight. The head of the pigs was split longitudinally and the atrophic changes of the turbinates, ethmoid bone, nasal septum and turbinate bones were rated with one to four crosses depending on their severity (Szabó and Antal, 1971). The average was calculated for the 20 pigs examined in the different groups as follows: e. g. 15–, 4 +, 1 ++ = 6 +, divided by 20 = 0.3 +.

Results

By determining the biological properties of numerous *B. bronchiseptica* strains isolated from healthy swine herds, several avirulent, atoxic strains were selected. The capsule of the strain used in the present experiment contained 5 out of the 7 K antigen factors (7, 8, 9, 12 and 13). The 18-h broth culture of this strain, in a dilution of 1 : 64, gave ++ agglutination with sheep RBCs; however, it failed to agglutinate calf and horse RBCs. During the determination of virulence, mice inoculated with any dilution of the bacterial culture survived for 7 days (in the lowest dilution this meant 10^7, 10^8 and 5×10^8 CFU in the case of intracerebral, intratracheal and intraperitoneal infection, respectively). In studies on exotoxin production, after intravenous inoculation with the lowest dilution no mice died within 6 days. The relative spleen mass of mice killed on day 7 (on average of 4 mice) was 103.5, 109, 106.5, and 118 mg per 10 g body mass if the 1 : 2, 1 : 4, 1 : 8, and 1 : 16 dilution of the bacterial culture was used, respectively. The spleen of the untreated control mice was 45.5 mg/10 g body mass.

The K antigen factors, the virulence and haemagglutinating ability of the strain did not change during passages aimed at determining its pathogenicity to swine. During the study of virulence, even mice inoculated with the lowest dilution of the 18-h broth culture survived. In studies on exotoxin production, the relative spleen mass of mice inoculated with the lowest (1 : 2) dilutions of the bacterial culture was found to be 109 mg/10 g body mass. In mice inocula-

ted with the 1 : 4, 1 : 8 and 1 : 16 dilution the relative spleen mass was 107, 117.5 and 114 mg per 10 g body mass, respectively.

Gross and histopathological examination of the intranasally infected SPF piglets revealed no pathological changes in the nose and other organs.

Bacteriological examinations performed in the immunized pig herds showed that up to day 7 the vaccine strain could be reisolated from the nasal cavity of all piglets. At 21 days old 10–70% of the piglets were infected by *B. bronchiseptica*. Examination of the subcultures made from the isolates for virulence and exotoxin production indicated that the isolates were mixtures of the vaccine strain and virulent *B. bronchiseptica* strains. Reisolation attempts performed on day 40 were successful only from the nasal mucosa of piglets vaccinated according to scheme III: the isolate always proved to be the vaccine strain. On day 90 virulent *B. bronchiseptica* strains were isolated

Table I

Results of bacteriological examination of the nasal cavity in the four herds (20 piglets per group)

Age of piglets (days)	Scheme of vaccination								Negative control	
	I		II		III		IV			
	B	P	B	P	B	P	B	P	B	P
7	20*	—	20*	—	20*	—	20*	—	16–19***	—
21	8**	9	8–14**	4–8	2–7**	5–7	4–8**	6–7	18–20***	9–14
40	0	7	0	3–6	9–13*	3–5	0	2–6	17–20***	8–17
90	3***	10	0	3–7	0	3–6	0	2–4	17–19***	10–13
120	8***	9	4–7***	4–9	0	5–6	0	6–8	17–18***	7–16

B = *B. bronchiseptica;* P = *P. multocida;* *: the isolate is the vaccine strain; **: the isolate is a mixture of the vaccine strain and a virulent strain; ***: the isolate is a virulent *B. bronchiseptica* strain. Figures indicate the number of strains isolated from the nasal cavity of 20 pigs; ranges show the extreme values of the number of strains isolated in the three herds

Table II

Immunoglobulin titres of the nasal secretion after vaccination according to scheme III and in the control animals in herds A, B and C (extreme values; 3 piglets per group)

Age of piglets (days)	Vaccinated piglets				Control piglets			
	B. bronchiseptica		*P. multocida*		*B. bronchiseptica*		*P. multocida*	
	IgA	IgG	IgA	IgG	IgA	IgG	IgA	IgG
7	0–50	50–100	—	50	50–100	100–200	—	—
21	200–400	400	100–200	200–400	100–200	100–400	50–100	100–200
40	400	400–800	100–200	200–400	200–400	400	50–100	100–400
90	400–800	800–1600	200–400	400	100–400	400–800	200	200–800
120	400–800	400–800	200–400	400	200–400	200–800	200	400–800

Table III

Pathological changes of the turbinates after local vaccination in the four swine herds (20 piglets per group)

Herds	Vaccination schemes I	II	III	IV	Negative control
	Average:	0.85	0.45		2.20
A		9—	12—		2—
		6+	7+		5+
		4++	1++		5++
		1+++			3+++
					5++++
	Average:		0.55	0.70	2.15
			12—	11—	3—
B			5+	4+	4+
			3++	5++	4++
					5+++
					4++++
	Average:	0.65	0.45	0.75	2.00
		10—	12—	8—	1—
C		7+	7+	10+	8+
		3++	1++	1++	4++
				1+++	4+++
					3++++
	Average: 0.85				1.9
	8—				4—
D	8+				3+
	3++				6++
	1+++				5+++
					2++++

from a low percentage of piglets immunized according to scheme I. By the age of 120 days, the infection rate had risen to 40%.

Bacteriological examination of the twice immunized piglets at 120 days old revealed infection by virulent *B. bronchiseptica* only in piglets immunized according to scheme II (infection rate: 20–35%). At that age *B. bronchiseptica* infection was absent from piglets immunized according to schemes III and IV.

P. multocida infection could be demonstrated first at 21 days old. By the age of 40 days piglets of all groups showed a reduced rate of infection in comparison with the controls: in the twice immunized piglets this was 10–30%. Infection rate underwent a slight increase with age; however, in piglets immunized according to scheme III or IV it did not exceed 25–40% even at 120 days old. The control animals showed a substantially higher infection rate during the experiment: between 21 and 120 days old it ranged between 35 and 85% (Table I).

Indirect IF aimed at demonstrating the immunoglobulin content of the nasal secretion. At 7 days old the nasal secretion of the experimental and control piglets was either devoid of IgA-class antibodies or contained such antibodies in a titre not exceeding 1 : 100. At the same age the titre of IgG-class antibodies fell between 1 : 50 and 1 : 200. The highest titres of IgA- and IgG-class antibodies were demonstrable in the nasal secretion of the immunized piglets at 40 and 120 days old, whereas in the control animals at 90 and 120 days old. The Ig titres of the control pigs' nasal secretion were usually by a dilution step lower. The IgA and IgG-class antibodies gave a reaction with both the *B. bronchiseptica* and *P. multocida* antigens. The titre of IgA-class antibodies reacting with *B. bronchiseptica* was 1 : 400–1 : 800 in the immunized piglets at 90 and 120 days old, whereas that reacting with *P. multocida* was between 1 : 200 and 1 : 400. At the same age the titres of IgG-class antibodies were 1 : 400–1 : 1600 and 1 : 400 with the *B. bronchiseptica* and *P. multocida* antigen, respectively (Table II).

Gross pathological examination of the pigs at slaughter. The 20 control pigs of herd A showed nasal lesions of 2.20 + severity on the average. In piglets vaccinated according to schemes II and III the severity of the nasal lesions was 0.85 + and 0.45 +, respectively.

The severity of nasal lesions in control pigs of herd B was 2.15 + on the average. In piglets vaccinated according to scheme III and IV the severity of the nasal lesions was 0.55 + and 0.70 +, respectively.

The severity of nasal lesions in control pigs of herd C was 2.00 + on the average. In piglets vaccinated according to schemes II, III and IV the severity of the nasal lesions was 0.65, 0.45 and 0.75 +, respectively.

The nasal lesions of 1.9 + severity, found in the control pigs of herd D, were reduced in severity to 0.85 + as a result of immunization according to scheme I (Table III).

Discussion

Secretory IgA is known to be the most important factor in the specific local antibacterial defence mechanism (Petzoldt, 1988): its level is in a closer correlation with protection against infection than are serum antibody titres. Secretory IgA production can be best elicited by local antigen application: if the antigen is given parenterally, repeated applications are needed for the production of even low levels of secretory IgA.

The possibility of local protection conferred by an avirulent *B. bronchiseptica* strain was indicated by the experiments of Harris and Switzer (1969). In the nasal mucosa freed from *B. bronchiseptica* by sulfonamide treatment a similar resistance developed against challenge with virulent *B. bronchiseptica* as after infection with the low-virulence strain. This resistance persisted for 95 days. Development of a live vaccine strain was facilitated by the results of

Éliás et al. (1985), who found that after chronic local application of *B. bronchiseptica* exotoxin no antibodies were demonstrable in the nasal secretion.

Therefore, it seemed justifiable to study the effect of applying an avirulent *B. bronchiseptica* strain on the nasal mucosa under large-scale conditions, in swine affected by AR.

In this study a natural avirulent mutant of *B. bronchiseptica* was used which had proved apathogenic to SPF piglets. The strain colonized the nasal mucosa and could be reisolated for 3 weeks. Studies aimed at determining the haemagglutinating ability showed, in accordance with that reported by Magyar et al. (1985), that the phase III *B. bronchiseptica* strain used had lost most of its haemagglutinin, as evidenced by its failure to agglutinate calf and horse RBCs. However, the strain did agglutinate sheep RBCs, showing that it had retained one of its haemagglutinins and part of its adhesive ability (Éliás and Krüger, 1988).

Bacteriological examinations performed in the immunized swine herds revealed that local immunization of newborn piglets before they could contract infection from the sows resulted in a mucosal immunity sufficiently strong to prevent colonization of the nasal mucosa by virulent *B. bronchiseptica* strains (Table I). Two immunizations provided the piglets with a mucosal immunity lasting 2–3 months. This is consistent with the findings of Wisecarver and Goodnow (1980) and Harris (1970). After local immunization with an avirulent *B. bronchiseptica* strain (D-1) Harris (1970) demonstrated local protection starting from day 25 after immunization and lasting 125 days.

It is surprising that a phase III strain which had lost one of its agglutinins and part of its pili and K antigens is able to induce firm protective immunity in the nasal mucosa. This may be attributed to the important protective role of antibodies produced upon exposure to somatic antigens. These antibodies cross-react with *P. multocida* antigens (Éliás et al., 1985). *P. multocida* infection was less severe in the vaccinated piglets than in the controls. This can be attributed partly to the cross-reaction between *B. bronchiseptica* and *P. multocida* strains, partly to the intact mucosa, free from damage done by *B. bronchiseptica*, which *P. multocida* strains can hardly colonize.

Studies aimed at determining the Ig content of the immunized pigs' nasal secretion have elucidated that both IgA and IgG-class antibodies play a role in the local immunity. It is remarkable that the immunized and the unvaccinated pigs have almost identical antibody levels in the nasal secretion at all ages. The essential difference is that mucosal protection induced by virulent *B. bronchiseptica* strains cannot prevent the development of regressive changes caused by exotoxins (Harris and Switzer, 1969).

The efficacy of the other immunization schemes varied. Two immunizations gave the best results, particularly if the first took place at birth and the other at 30 days old.

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LOCAL ACTIVATION OF THE MONOCYTE-MACROPHAGE SYSTEM IN EXPERIMENTAL CONTACT DERMATITIS OF THE GUINEA PIG

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Activation of the monocyte-macrophage (histiocyte) system was evaluated in guinea pig skin samples with experimental contact dermatitis. Changes in the cytoplasmic organelles of macrophages revealed by electron microscopy suggest an activation mechanism that could also be demonstrated light microscopically. Giant cells, the final transformation forms of macrophages, were recognizable 72 h after challenge in each skin-cytosmear of guinea pigs treated with the sensitizing agent dinitrochlorobenzene (DNCB) and Ipam 40. Since preparation of cytosmears is simple and rapid, it can usefully be integrated into various sensitizing tests to facilitate evaluation of the sensitization responses.

Keywords: Monocyte-macrophage system, skin-cytosmears, light and electron microscopy, sensitization, guinea pig

Sensitizing contact dermatitis is one of the most important clinical manifestations of delayed hypersensitivity responses.

Among chemicals used in the industry, households and agriculture, there are compounds of low molecular weight which bind protein as haptenes and can induce sensitization in the living organism after repeated contact (Baer and Harber, 1971; Polak, 1978).

Screening of the sensitizing agents belongs to the field of safety evaluation, but the tests generally used (Buehler, 1965; Magnusson and Kligman, 1969) enable only macroscopic evaluation.

In a previous paper (Vermes et al., 1988) we reported a complex method for the evaluation of skin sensitizing tests and emphasized the importance of macrophages in the recognition of proliferative inflammatory responses.

This paper presents the morphological transformation of macrophages adaptive to the functional changes in experimental contact dermatitis with the aid of cytological and histological preparations.

Materials and methods

Contact dermatitis was experimentally induced in DPH albino guinea pigs by the subirritative dosages of dinitrochlorobenzene (DNCB) in 1 and 0.1% solutions and of the pesticide Ipam 40 (containing 40% ammonium-

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methyl-dithiocarbamate) in 20% solution according to the method of Draize (1959), using ethanol and distilled water vehicle controls.

Twenty-four, 48, 72 and 96 h after challenge, 18 sensitized and 6 control animals were evaluated on each occasion (total: n = 96). Skin samples were taken from the responding areas and cytosmears were prepared as described previously (Vermes et al., 1988).

For histology, skin fragments were fixed in 4% neutral formalin and embedded in paraffin. Sections, 5–7 μ thick, were prepared and stained with toluidine blue–eosin (Krutsay, 1980).

For electron microscopy, skin fragments were fixed in sodium cacodylate buffer, in a mixture of 25% glutaraldehyde and 37% formalin, rinsed with the buffer and post-fixed in 3% osmium tetroxide. After dehydration in ethanol, the samples were embedded in Durcupan-ACM (Fluka) resin, and sections were cut with an LKB Ultraton type ultramicrotome. The semithin sections were stained with toluidine blue and evaluated under light microscope. The ultrathin sections were counterstained in 5% uranyl acetate, then, after rinsing with distilled water, with Reynold's lead citrate solution.

Electron micrographs were prepared with a Tesla BS type electron microscope.

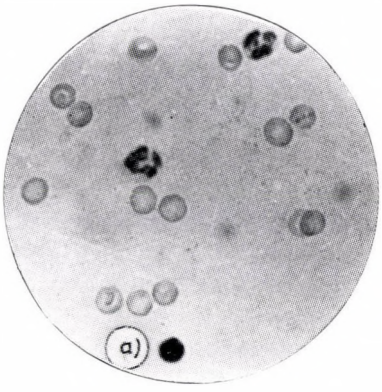
Results

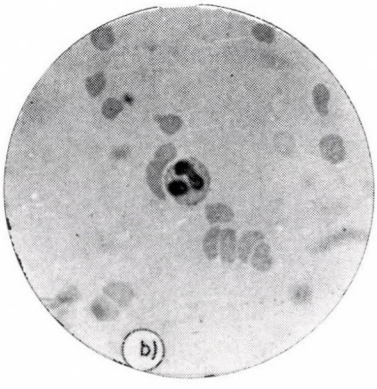
In the cytosmears neutrophilic and eosinophilic granulocytes, macrophages, and lymphocytes were recognized and counted. Twenty-four h after challenge, the macrophages appeared similar in shape and size to the monocytes visible in the peripheral blood films. The nucleus was most frequently crescent-shaped, and the cytoplasm was homogeneous with slightly undulating surface. By the 48th h the nucleus and the cytoplasm had become abundant in filaments and the cytoplasm had vacuolar appearance. The cells showed more intense phagocytic activity. At this stage they were very similar in appearance to activated monocytes in tissue cultures (Laskin and Pilaro, 1986).

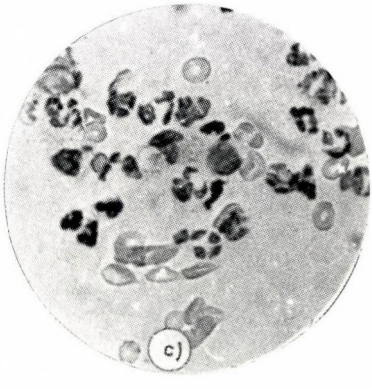
By the 72nd h, the size of macrophages approximated 30–50 μ. Their fusion resulted in multinucleated cells. Then, 96 h after challenge, the macrophages decreased in number, the cell body became elongated with frequent formation of tail-like pseudopodia (Fig. 1).

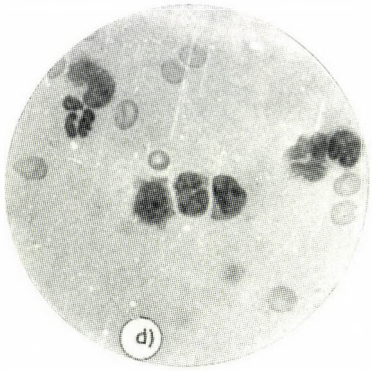
The light microscopic sections clearly demonstrated the migration pattern of macrophages. Their mass emigration from the peripheral blood took place 24 h after challenge and they took an elongated shape to facilitate penetration through the endothelial cells (Fig. 2). At the 72nd h, macrophages were predominant within the entire responding skin area (Fig. 3), and were enlarged due to activation.

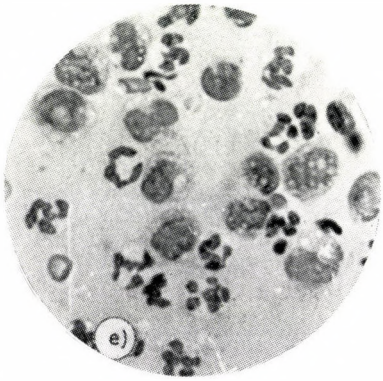
The electron micrographs revealed that at the challenge site the skin contained numerous macrophages activated from monocytes.

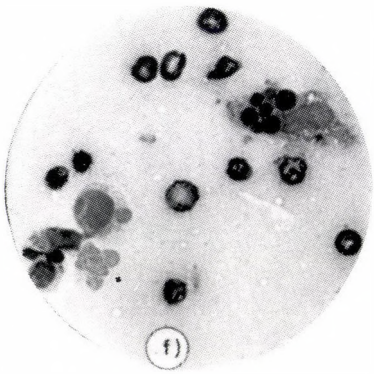

a)

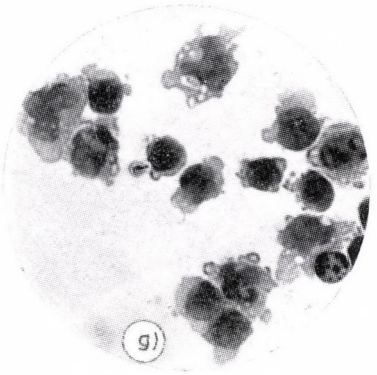

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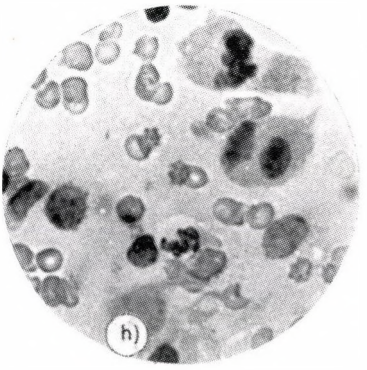

c)

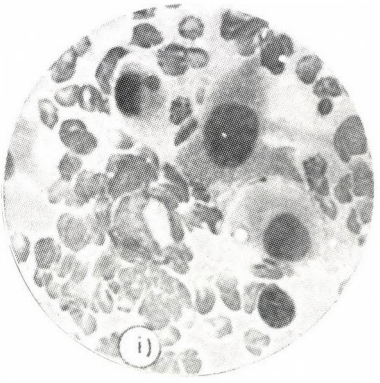

d)


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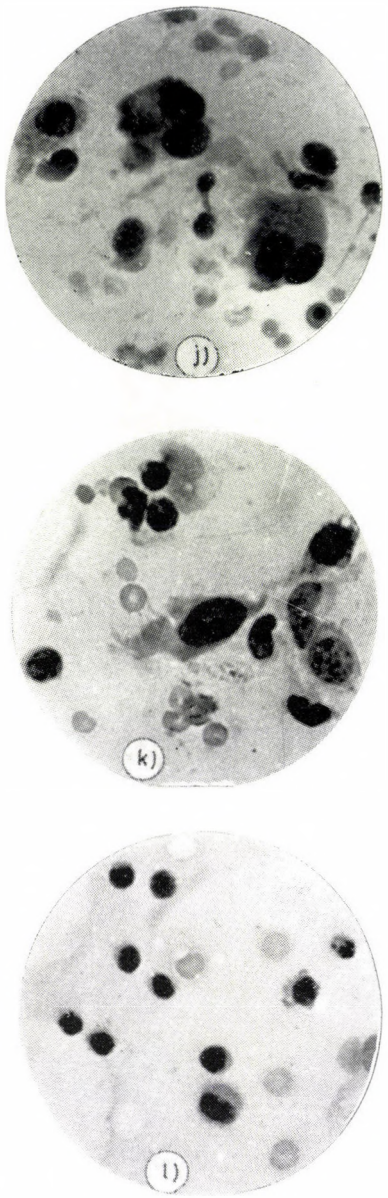


Fig. 1. Activation of the monocyte-macrophage system in skin cytosmears from guinea pigs at various intervals after challenge. A, B: ethanol control cytosmears 24 and 72 h after challenge, respectively; C, D: 24th h: cytosmears showing abundance of cells and monocytes, in response to ec. treatment with 1% DNCB (C) and 20% Ipam 40 (D), respectively; E, F, G: 48th h: cytosmears showing vacuolated cells (E), phagocytosed red blood cells (F) and filamentous cells (G), in response to ec. treatment with 1% DNCB (E, F) and 20% Ipam 40 (G) resp.; H, I, J: 72th h: cytosmears showing mitotic activity (H), swelling of plasma and nucleus (I), and multinucleated cells (I), in response to ec. treatment with 1% DNCB (H) and 20% Ipam 40 (I, J) respectively; K, L: 96th h: decreased macrophage activity (K) and predominance of lymphocytes (L) in response to ec. treatment with 1% DNCB (K) and 20% Ipam 40 (L), respectively

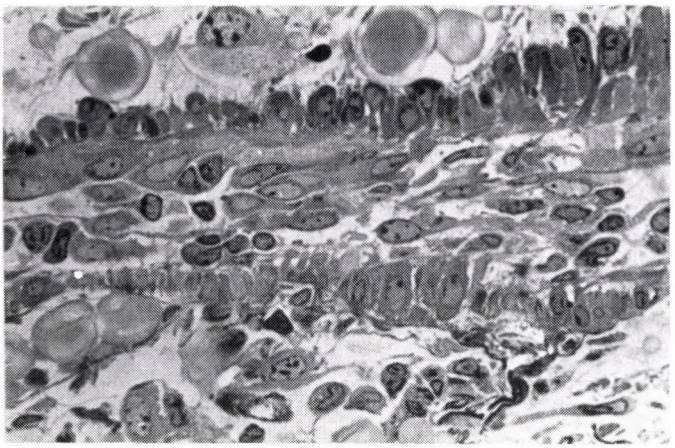


Fig. 2. Emigration of inflammatory cells from the capillary in response to ic. treatment with 0.1% DNCB, 24 h after challenge (Semithin section, toluidine blue staining, ×1000)

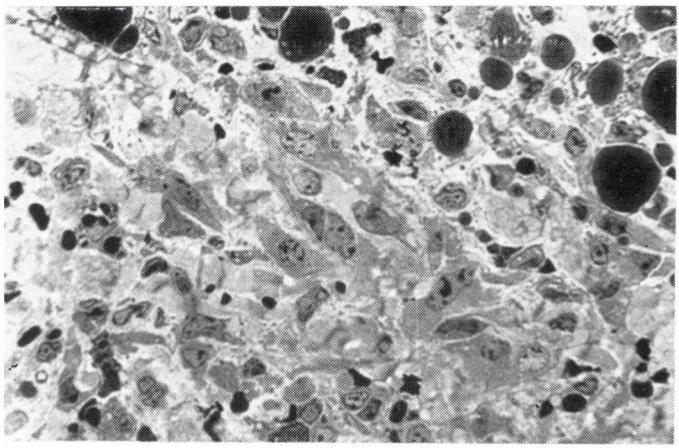
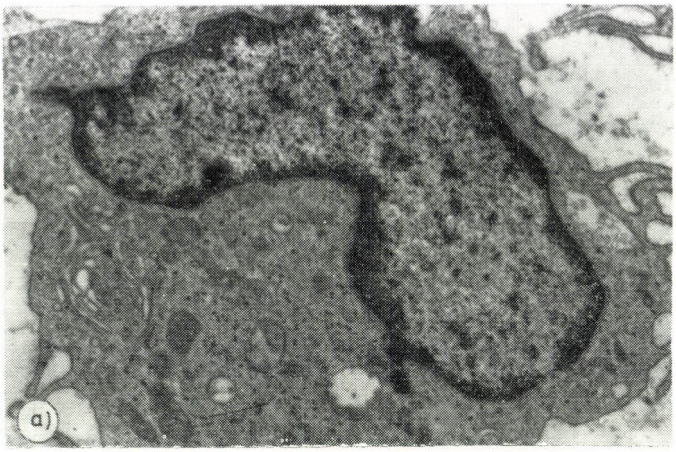
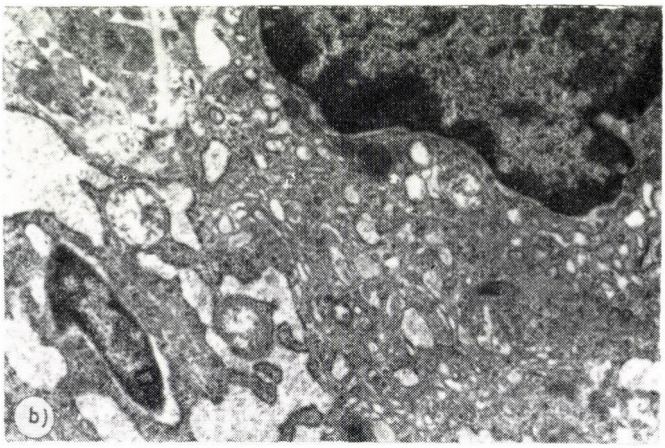
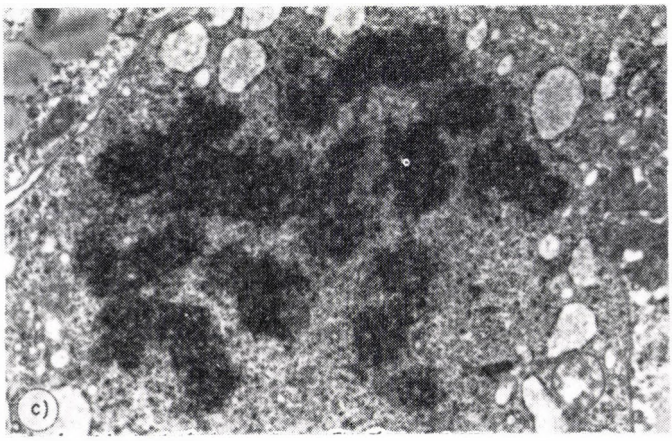


Fig. 3. Infiltration of inflammatory cells in the subcutis in response to ic. treatment with 0.1% DNCB, 72 h after challenge (Semithin section, toluidine blue staining, ×1000)


a)


b)


c)

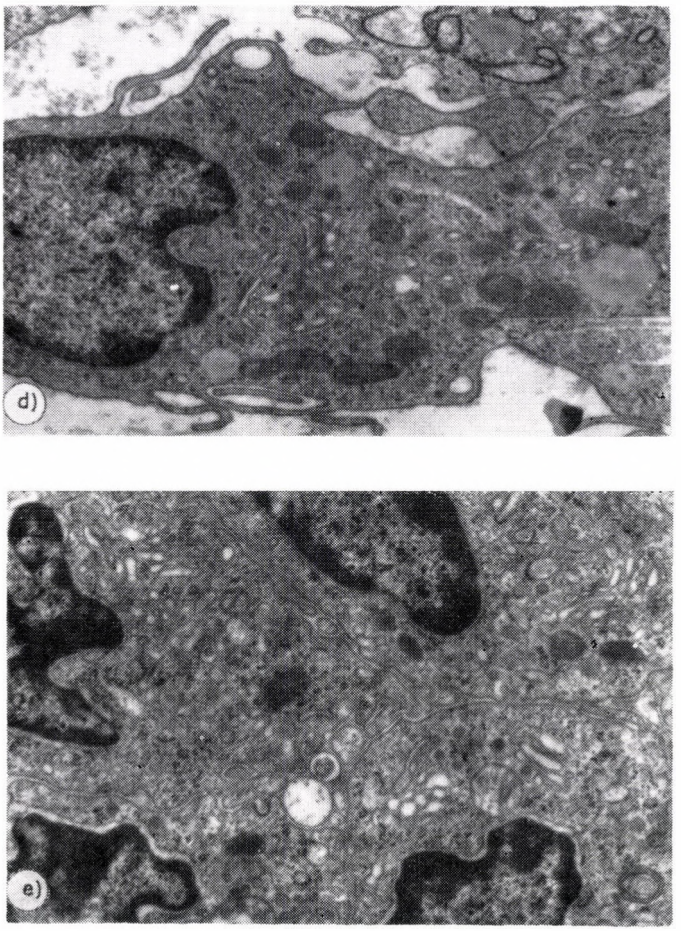


Fig. 4. Macrophage activation pattern in the guinea pig skin at various intervals after challenge. *A:* 24th h, activated monocyte; *B:* 48th h, macrophage; *C:* 72nd h, proliferation, mitosis; *D:* 72nd h, epitheloid cell; *E:* 72nd h, giant cell. Corresponding treatments: A, B = 1% DNCB ec.; C–E: 0.1% DNCB ic. ×6000

These cells were round-shaped with several pseudo-cytoplasmic filaments, and their nuclei were segmented in appearance. The cytoplasm contained numerous RER and mitochondria, frequently with a centriolum around the Golgi-zone. The presence of vacuoles, lamellary phagosomes, lysosomes, red blood cells, and cellular debris indicated progressive phagocytosis. The cells showed increased mitotic activity 72 h after challenge.

Maturation of macrophages resulted in the formation of epitheloid cells varying in size and having an elongated slipper-like shape. Their nuclei contained little chromatin and occasionally several nucleoli and a small incisure on the surface.

In the cytoplasm of the epitheloid cells the most characteristic findings included the multiple Golgi-region, vacuolation, abundance of cytoplasmic filaments, and intense protein synthesis.

Mitochondria were prominent, and the cells were highly phagocytic.

Finally, we also noted the presence of giant cells associated to localize and eliminate antigens. These cells resulted from the fusion of epitheloid cells (Fig. 4).

Discussion

Monoblasts and monocytes originating from the bone marrow cells may leave the peripheral blood, enter the tissues and transform into macrophages. Some of these macrophages exert an antigen-oriented immuno-phagocytic activity regulated by the T lymphocytes. As a result of further stimulation these macrophages are transformed into epitheloid cells and ultimately fuse to form multinucleated giant cells (Spector, 1977; Sutton and Weiss, 1966).

In the present experiments sensitization-induced maturation of cells of the macrophage system was monitored.

The cytosmears revealed an increase of macrophages not only in number but in relative proportion as well as in participation in the inflammatory response. Formation of cytoplasmic filaments, enlargement of cells with intense mitotic activity, and presence of giant cells were noted.

The light microscopic slides proved suitable for detecting migration, enlargement of macrophages and their increase in number.

The electron micrographs demonstrated the ultrastructural changes of macrophages including increased number of ribosomes and mitochondria, prominent Golgi complex, abundance of lysosomes and vacuoles, intense phagocytosis and, finally, formation of giant cells, which are all indicative of activation. With knowledge of the ultrastructural changes we assume that morphological transformation of macrophages is a general consequence of immunostimulation.

Naturally, it is not necessary to carry out electron microscopic studies, but evaluation of skin-cytosmears can usefully be integrated in the various

sensitization tests. When skin-cytosmears contain macrophages of varying size and structure in abundant number and with mitotic activity, especially with the presence of multinucleated giant cells, it is probable that the test animal has been exposed to a sensitizing agent.

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ELECTROPHORETIC STUDY OF LAYING HENS' SERUM BY CROSSED IMMUNOELECTROPHORESIS

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(Received October 19, 1987)

Serum from hens in full egg production was examined by crossed immunoelectrophoresis. The picture obtained was in general similar to that found for chickens. The prealbumins, albumin, glycoproteins, haemopexin and alpha-2-macroglobulin as well as transferrin, IgG, IgM and C3 complement β-1-A β-1-C globulin were readily identified. The characteristic feature was the presence of lipoprotein complex (LPC) in the gamma-globulin region and lipoprotein (LP) in the beta-globulin region. The slow moving lipoprotein (9) was detected in the alpha-beta-gamma-globulin area. Transferrin was found at higher level. Some tentative correlations of these precipitates with results published by various workers are discussed.

Keywords: Laying hen, serum, crossed immunoelectrophoresis

Laurell's crossed immunoelectrophoresis has been used in the study of serum proteins in growing chickens (Singh, 1978, 1981). This communication describes some observations on serum proteins of laying hens.

Materials and methods

Pooled serum from White Leghorn hens in full egg production was used.

Crossed immunoelectrophoresis was done essentially as described (Weeke, 1973). One per cent agarose type HSA (Litex A/S, Glostrup, Denmark) in Tris/barbital buffer (containing 0.073 M Tris, 0.024 M barbital, 0.00034 M calcium lactate and 0.0019 M sodium azide), pH 8.6, was used. Two μl of laying hen serum was run for 1.5 h at 10 Volts/cm in the first dimension. Second dimension was performed in the presence of rabbit anti-chicken (whole) serum (Dakopatts A/S, Copenhagen), corresponding to 12.5 μl antibody/cm^2 of gel, and run for 18–20 h at 2 Volts/cm. The plates were pressed, washed and dried, and stained with Coomassie Brilliant Blue R-250. The immunoprecipitates were determined as previously described (Singh, 1978) by

(i) Morphology;

(ii) Electrophoretic position;

(iii) Using specific antibodies in intermediate gel in crossed immuno-

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electrophoresis (Axelsen, 1973). The specific antibodies used were anti-human prealbumin, anti-human transferrin, anti-chicken IgG and anti-chicken IgM.

(iv) Specific staining methods: Oil Red O staining (Uriel, 1964) for lipoproteins and periodic acid–formazan reaction (Uriel, 1964) for glycoproteins.

The numbering of precipitates (arcs) was given anodically as described (Singh, 1978).

Results and discussion

Fig. 1 illustrates the electrophoretic picture of laying hens' serum. The pattern obtained was in general similar to that reported in chickens (Singh, 1978). The prealbumins, albumin, arcs 5, 7, 8, 10, 13, 21 and 24, and alpha-2-macroglobulin as well as transferrin, IgG, IgM and C3 complement β-1-A, β-1-C globulin were readily identified. Figs 2 and 3 give the identification of two of the proteins by crossed immunoelectrophoresis with intermediate gel. However, the mobility and the number of precipitates of alpha-beta-globulin regions appeared to be different from those of chickens. Besides, the precipitate marked as LPC was detected in the gamma-globulin region.

Component 9 was a heavy lipid stained precipitate as determined by staining with Oil Red 0 (Uriel, 1964) and was seen in the alpha-beta-gamma globulin regions. The migration of this component was much slower than that of the alpha-1 lipoprotein in growing chickens (Singh, 1978 and 1981). It was also found considerably decreased in height. Its trailing edge (cathodic) was seen crossing the leading edge (anodic) of the LPC component in the gamma-globulin region. However, its apparent position (anodic) and distinctiveness appeared to be not very dissimilar to that of alpha-1 lipoprotein in chickens. This precipitate is probably similar to the lipovitellin in laying females as reported by Schjeide (1963).

The lipoprotein LP was a symmetric precipitate poorly stained with Oil Red 0, and it migrated in the alpha-2-beta-globulin regions.

It is of interest to note that no precipitate occurred in laying hens' serum in the position of beta-lipoprotein as seen in chickens (Singh, 1978 and 1981) or with morphology alike beta-lipoprotein, by double lipid-protein staining.

Moore (1948) demonstrated the presence of a lipoprotein component in the serum of laying hens. There was also an increase in lipoprotein of laying hens in the beta-globulin region and a decrease in the alpha-1-globulin region. This picture was confirmed in general by Brandt et al. (1951), Clegg and Hein (1953), McKinley et al. (1953) and Vanstone et al. (1955), using electrophoretic studies in hens. The work of McIndoe (1959) also indicated a similar situation.

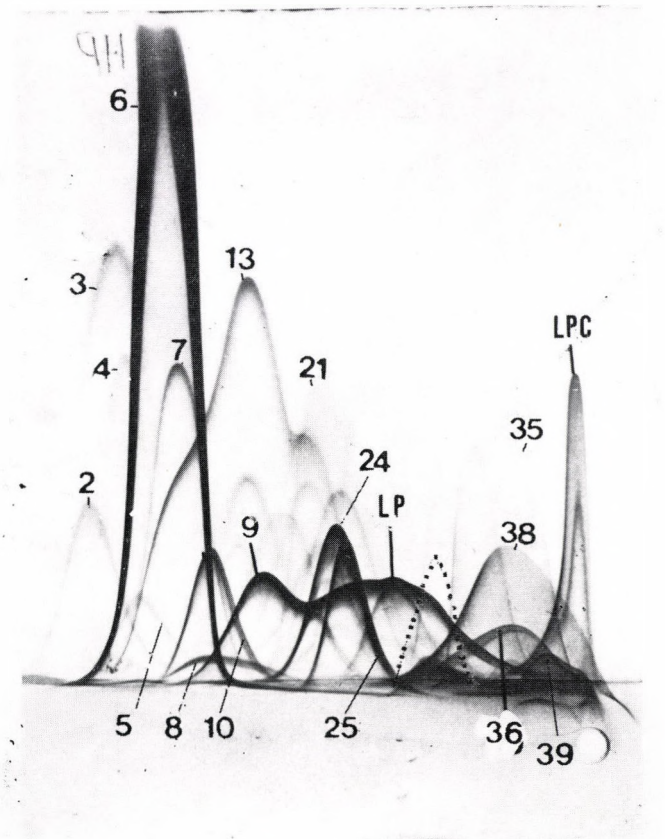


Fig. 1. Electrophoretic picture of laying hens' serum examined by crossed immunoelectrophoresis. Anode to the left. Arcs 2, 3 and 4: prealbumins; arc 5: double contoured, weak precipitate; arc 6: albumin; arc 7: symmetric, distinct precipitate; arc 8: glycoprotein; arc 9: lipoprotein; arcs 10 and 13: glycoproteins; arc 21: haemopexin; arc 24: glycoprotein; arc 25: alpha-2-macroglobulin; arc LP: lipoprotein; arc 35: transferrin; arc 36: IgM; arc 38: IgG; arc LPC: lipoprotein complex. Note: the other precipitates (although not numbered) appeared similar in mobility and morphology to those reported in growing chickens (Singh, 1978)

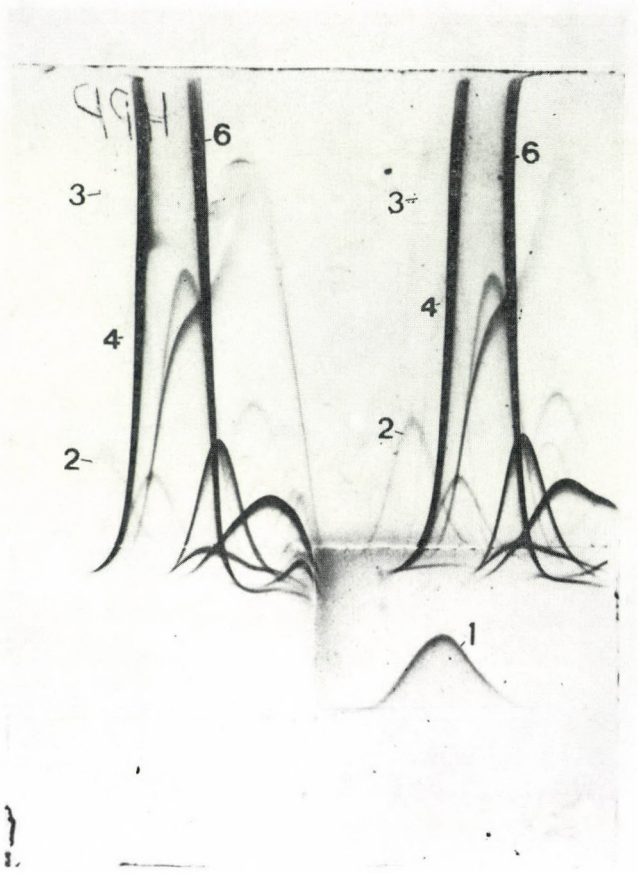


Fig. 2. Crossed immunoelectrophoresis with intermediate gel showing arc 1 obtained with anti-human prealbumin. Anode to the left. The control without anti-human prealbumin is shown in the left part of the figure

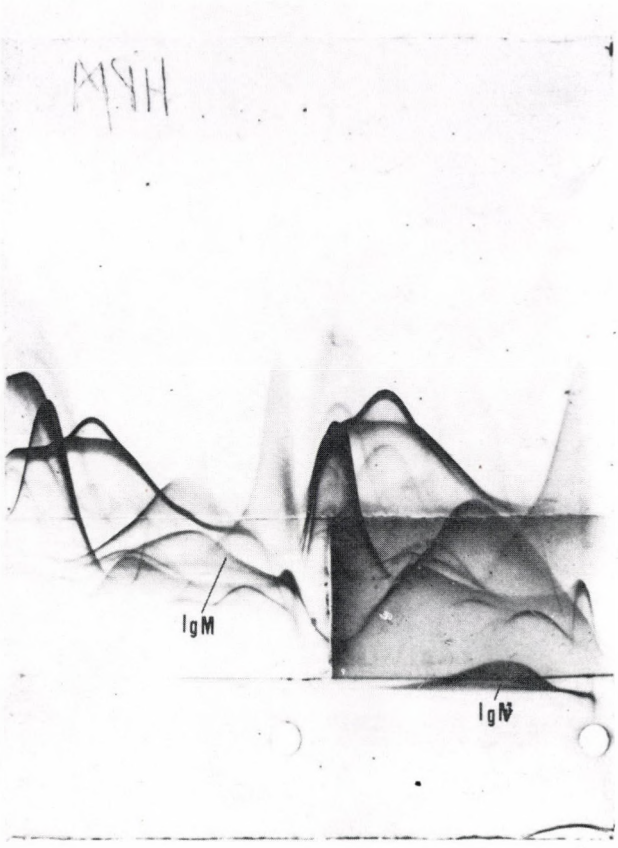


Fig. 3. Crossed immunoelectrophoresis with intermediate gel showing the identification of IgM with anti-chicken IgM (anti-IgM used was not anti-μ and therefore reacted with IgG also). Anode to the left. The control without anti-chicken IgM is shown in the left part of the figure

Evidently, the observations made on serum lipoproteins in the current study fairly agree with those of the various authors cited.

A component which was present as a distinct precipitate (indicated by a dotted arc in Fig. 1) in fresh serum from laying hens disappeared when the serum was stored at —20 °C for two days and frozen and thawed once. The precipitate migrated in the beta-gamma-globulin regions. This component was also detected in fresh chicken serum in the same position (Singh, unpublished data).

Transferrin was detected at higher level in serum from laying hens. This finding is in conformity with the works of Ramsay and Campbell (1954) and Planas et al. (1961) who found that laying females showed higher iron-binding capacity in the serum in order to prevent losses of iron through eggs.

In other serum proteins no significant differences were recorded between hens in lay in the current study and growing chickens (Singh, 1978 and 1981).

Acknowledgement

This work was carried out during the tenure of a DANIDA Scholarship. The anti-human prealbumin and anti-human transferrin were a gift from Dakopatts A/S, Copenhagen, and goat anti-chicken IgG and anti-chicken IgM from Dr F. C. McDuffie (Mayo Clinics, U. S. A.).

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STUDY OF THE METABOLISM AND EXCRETION OF T-2 TOXIN, A TRICHOTHECENE FUSARIOTOXIN, IN RABBITS

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Acute and subacute toxicity studies were performed on 18 and 26 rabbits (body mass: 2 kg), respectively, treated with T-2 toxin per os. The T-2 toxin, HT-2 toxin, neosolaniol, T-2 triol and T-2 tetraol concentrations of the stomach contents, caecal contents, faeces, and liver were determined. The results are summarized in tables.

The toxin concentration found in the stomach and caecal contents and faeces was in linear correlation with the ingested amount of T-2 toxin. Toxin concentration was higher in the posterior portions of the intestine, indicating that the main route of excretion was via the faeces. From the chemical analysis of the faeces the toxin load could be estimated. Oral administration of T-2 toxin over a period of 10 days at a subtoxic dose rate (1 mg/kg body mass/day) revealed a gradual decrease in the toxin-metabolizing capacity of the liver, as indicated by the increasing amount of T-2 toxin per unit of liver tissue and by the decreasing amount of metabolites. Parallel to this, the susceptibility of the organism to the toxin increased.

Keywords: T-2 toxin, trichothecene, fusariotoxin, metabolism, excretion, rabbit

Like other mycotoxins, T-2 toxin is chemically stable and resists the action of external factors. Recently, however, it has come to light that in a biologically active environment the toxin is metabolized (decomposed or transformed) rather quickly. Wei et al. (1971) reported that in a slightly alkaline medium T-2 toxin is hydrolysed into HT-2 toxin, T-2 triol and T-2 tetraol. The 12, 13-epoxy group itself remains intact. In *Fusarium nivale* and *F. solani* cultures Yoshizawa and Morooka (1975) demonstrated, in addition to T-2, also HT-2 toxin. A similar derivative was isolated from T-2 solution treated with human and bovine liver homogenate (Ohta et al., 1977; Ellison and Kotsonis, 1974).

Robison et al. (1979) and Mirocha and Robison (1976) studied the metabolism of T-2 in 8–11 weeks old broiler chickens. About half of the excreted toxin quantity was T-2 toxin, while neosolaniol, HT-2 toxin and T-2 tetraol constituted the other half. The metabolizing capacity of the liver varies by animal species (Ohta et al., 1978). Trichothecene mycotoxins including T-2 toxin are metabolized in embryonated hen's eggs (Bata et al., 1983) and bacterial cultures (Ueno et al., 1983) as well. Furthermore, *F. sporotrichioides*,

the strain which produces T-2, catabolizes the toxin parallel to synthesizing it (Sándor et al., 1984).

There is a scarcity of data in the literature on the metabolism of T-2 in rabbits. Lucisano et al. (1983) performed subacute toxicity studies of T-2 in rabbits, but failed to study the metabolism of the toxin. In preliminary experiments we found that T-2 is metabolized also in rabbits, but less rapidly than in other animal species (Ványi et al., 1986; Ványi and Bata, 1987). The rabbits excrete the toxin in the faeces and to a lesser extent in the urine. T-2 toxin and its metabolites are excreted in the milk as well.

In Hungary table rabbit and angora wool production has great economic importance. The digestive mechanism of the rabbit differs from that of all other monogastric domestic animal species. This difference is due to the fact that rabbits consume high-fibre diets and practise coprophagy. These facts prompted us to get a closer insight into the metabolism of T-2 toxin in, and its excretion from, the rabbit's organism.

Materials and methods

Toxin production for analytical standard

To produce T-2 toxin, HT-2 toxin and neosolaniol, *F. sporotrichioides* was grown on rice in three successive temperature-periods: at 22 °C for 7 days, at 4 °C for 7 days and at 22 °C for 10 days. The culture was dried, ground and extracted with acetonitrile-water (5 : 1). The acetonitrile extract was partitioned with hexane. The concentrated acetonitrile phase was purified on silica gel column (45 mesh) by elution of a step gradient system consisting of benzene and acetone. The fractions containing HT-2 toxin or neosolaniol were purified further by preparative thin-layer chromatography (TLC), using chloroform–ethanol (8 : 2) mixture. Each toxin was crystallized repeatedly from ethyl acetate–hexane. Each product was of 95–97% purity as determined by gas-liquid chromatography (GLC).

T-2 tetraol and T-2 triol toxins were obtained by hydrolysis of HT-2 toxin. HT-2 toxin was dissolved in 0.5 M NaOH in methanol and stirred at room temperature for 1 h. The solvent was removed *in vacuo*. The products were purified by preparative TLC and crystallized repeatedly from ethyl acetate–hexane. The T-2 tetraol and T-2 triol used in this study were of 90–92% purity, controlled by GLC.

Chemical analysis

Chemical analysis was performed by capillary gas chromatography as described earlier (Ványi et al., 1982). The samples were treated with glucuronidase and extracted with ethyl acetate. After evaporation, the extract was

defatted using a polar-apolar partitioning system, purified on a Lichroprep RP 18 column and, after derivation, analysed on a capillary column pretreated with CB 8 humidifier.

Qualitative evaluation was performed on the basis of the retention time. After transmethylation, another analysis was performed for confirmation. Quantitative evaluation was based on the area under the curve.

Rabbit experiments

Acute toxicity study. Growing rabbits of 2 kg body mass were used in the experiments according to the following design.

Number of rabbits	Toxin dose per animal*	Toxin dose per kg body mass
2	2 mg	1 mg
2	4 mg	2 mg
2	8 mg	4 mg
2	12 mg	6 mg
2	16 mg	8 mg
2	20 mg	10 mg
2	30 mg	15 mg

* Administered through an oesophagogastric tube

Further two rabbits were treated with solvent (dimethyl sulfoxide) only, and two untreated rabbits served as controls.

Forty-eight hours after oral toxin administration the rabbits were killed by bleeding. From these rabbits, and from those that had died in the meantime, samples were taken from the stomach contents, caecal contents, and faeces for chemical analysis. The samples were assayed for T-2 toxin, HT-2 toxin, neosolaniol, T-2 triol, and T-2 tetraol by capillary gas chromatography.

Subacute toxicity study and study of toxin metabolism

Thirteen rabbits weighing 2 kg received 1 mg/kg body mass T-2 toxin mixed in the feed, daily. Another thirteen rabbits were treated with the toxin-free solvent and served as controls. One treated and one control rabbit were killed by bleeding 6 and 12 h after treatment and then at the same time daily. The stomach contents, caecal contents, faeces, and liver were assayed for toxin concentration.

Results and discussion

The toxin concentration of the stomach contents, caecal contents, and faeces, as determined in the acute toxicity experiment, is shown in Table I. The toxin concentration is proportional to the amount of toxin ingested. The T-2 concentration of the stomach contents, T-2 concentration of the caecal contents, and the toxin concentration of the faeces are in a close linear correlation with the amount of T-2 toxin ingested.

It can be seen from Table I that proceeding towards the end of the digestive tract the amount of T-2 toxin increases.

The T-2 toxin, HT-2 toxin, neosolaniol, T-2 triol and T-2 tetraol concentrations of the stomach contents, caecal contents, faeces and liver, as determined in the subacute toxicity experiment, are shown in Tables II, III, IV and V.

From Tables I and II it appears that the ingested T-2 toxin remains unchanged in the stomach and is passed on unaltered. In the stomach contents practically no metabolites of T-2 toxin can be detected. On the other hand, in

Table I

Toxin concentrations measured in animals of the acute toxicity experiment (mg/100 g) 48 h after oral toxin administration

Toxin ingested (mg/kg)	T-2 toxin	HT-2 toxin	Neosolaniol	T-2 triol	T-2 tetraol
			Stomach contents		
1	0	0	0	0	0
2	0.2	0.1	0	0	0
4	0.5	0.1	0	0	0
6	0.8	0	0	0	0
10	3.8	0	0	0	0
15	6.3	0.2	0	0	0
			Caecal contents		
1	0.6	0.1	0.2	0.1	0
2	1.4	0.3	0.3	0.2	0
4	3.2	0.6	0.6	0.6	0
6	4.5	0.7	0.6	0.6	0.1
10	5.6	0.5	0.5	0.4	0.1
15	10.3	0.1	0.1	0	0
			Faeces		
1	3.2	2.3	1.9	1.5	
2	4.8	3.2	3.1	2.4	0
4	7.2	4.7	4.2	3.2	0.4
6	9.2	5.7	4.3	3.0	0
10	12.2	3.5	2.1	2.3	0
15	10.0	1.2	0.7	0.3	0

Table II

Toxin concentration of the stomach contents (mg/kg) in rabbits treated with 1 mg/kg body mass T-2 toxin daily

Time of sampling (h after the first treatment)	Toxin or metabolite				
	T-2 toxin	HT-2 toxin	Neo-solaniol	T-2 triol	T-2 tetraol
0	0.3	0	0	0	
6	0.7	0.1	0	0	
12	0.9	0	0	0	
24	1.2	0.1	0.05	0	0
48	1.4	0.2	0.1	0	0
72	1.2	0.1	0.5	0	0
96	1.0	0	0	0	0
120	1.1	0	0	0	0
144	1.0	0	0	0	0
168	0.9	0	0	0	0
192	0.8	0	0	0	0
216	0.9	0	0	0	0
240	0.9	0	0	0	0

Table III

Toxin concentration of the caecal contents (mg/kg) in rabbits treated with 1 mg/kg body mass T-2 toxin daily

Time of sampling (h after the first treatment)	Toxin or metabolite				
	T-2 toxin	HT-2 toxin	Neo-solaniol	T-2 triol	T-2 tetraol
0	0	0	0	0	0
6	1.3	0.6	0.4	0.1	0
12	1.6	0.4	0.3	0.2	0
24	1.8	0.3	0.3	0.2	0
48	1.6	0.3	0.3	0.2	0
72	1.7	0.4	0.4	0.2	0
96	1.7	0.3	0.4	0.3	0
120	1.8	0.4	0.3	0.2	0
144	1.7	0.4	0.3	0.3	0
168	1.9	0.3	0.3	0.2	0
192	1.7	0.3	0.2	0.2	0
216	1.8	0.3	0.2	0.1	0
240	1.7	0.3	0.1	0.2	0

the caecal contents (Tables I and III) all metabolites of the toxin are demonstrable. The same holds for the faeces (Tables I and IV, Fig. 1), with the exception that in the latter the quantity of metabolites usually significantly increased (that of T-2 tetraol only in two samples). In rabbits receiving 10 or 15 mg toxin per kg body mass, the rate of toxin degradation is insignificant. These data suggest that in the rabbit caecum the intensive digestive processes are accompanied by substantial toxin catabolism. This toxin-degrading capacity, however, seems to get exhausted around a toxin dose of 4–5 mg/kg body

mass. Namely, in animals treated with 6 mg/kg body mass the proportion of metabolites does not rise further. In animals receiving even higher doses (10 or 15 mg/kg body mass) the proportion of metabolites decreases, indicating that the bulk of the T-2 toxin is excreted in the faeces unchanged.

The toxin and metabolite concentrations measurable in the liver (Table V, Fig. 2) deserve particular attention. The 1 mg/kg body mass toxin dose ingested daily in the first three days is mostly metabolized in the liver, and only about one-fourth remains unchanged. Beyond day 3 the increased toxin load results in a reducedt oxin-metabolizing capacity of the liver. On day 10 more

Table IV

Toxin concentration of the faeces (mg/kg) in rabbits treated with 1 mg/kg body mass T-2 toxin daily

Time of sampling (h after the first treatment)	Toxin or metabolite				
	T-2 toxin	HT-2 toxin	Neo-solaniol	T-2 triol	T-2 tetraol
24	1.6	1.4	0.6	0.5	0
48	2.2	1.6	0.7	0.6	0
72	2.8	1.2	0.5	0.5	0.1
96	2.0	1.5	0.7	0.4	0
120	2.2	1.4	0.8	0.5	0
144	2.4	1.4	0.8	0.6	0
168	2.2	1.6	0.6	0.4	0
192	2.0	1.5	0.5	0.4	0
216	2.4	1.5	0.4	0.3	0
240	2.6	1.0	0.3	0.2	0

Table V

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

Time of sampling (h after the first treatment)	Toxin or metabolite				
	T-2 toxin	HT-2 toxin	Neo-solaniol	T-2 triol	T-2 tetraol
0	0	0	0	0	0
6	40	160	140	80	0
12	70	140	140	90	0
24	80	120	130	80	+
48	120	160	140	120	0
72	140	140	140	140	+
96	180	130	120	100	0
120	240	110	100	80	0
144	280	90	80	80	0
168	320	90	70	60	0
192	340	80	60	40	0
216	360	40	40	40	0
240	360	40	20	20	0

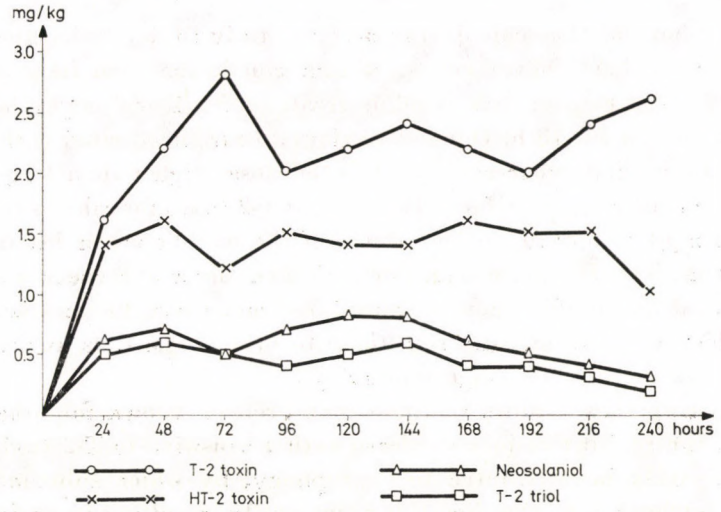


Fig. 1. Toxin concentration of the faeces (mg/kg) in rabbits treated with 1 mg/kg body mass T-2 toxin daily

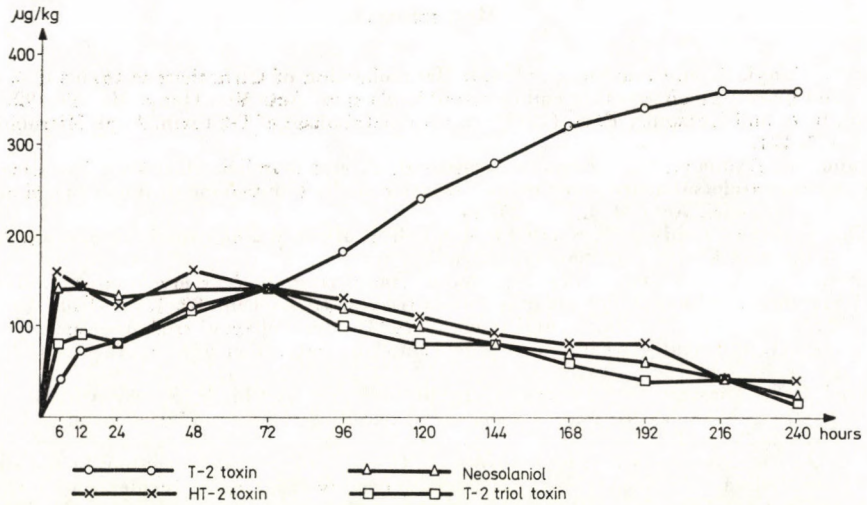


Fig. 2. Toxin concentration of the liver (µg/kg) in rabbits treated with 1 mg/kg body mass T-2 toxin daily

than 80% of the toxin quantity demonstrable in the liver is present as unchanged T-2 toxin. The metabolism of T-2 toxin causes a marked reduction in its biological activity. Thus, if the bulk of the toxin leaves the liver unchanged, the organism will have higher relative sensitivity to it. Prolonged administration of the toxin enhances the organism's sensitivity. The relationship between the duration of toxin administration and the increase in sensitivity is much stronger than linear.

It was beyond the scope of the present study to determine the LD_{50} or other toxicity values. Nevertheless, certain conclusions can be drawn from the results in that respect, too. Rabbits treated with 1 or 2 mg/kg body mass T-2 toxin survived for 48 h. One out of the two rabbits treated with 4 mg/kg body mass toxin died, whereas all rabbits fed doses higher than 4 mg/kg body mass died. Considering these facts, the LD_{50} of T-2 toxin for rabbits is probably around 3–4 mg/kg body mass. The results of the metabolic studies are consistent with this. The T-2 toxin quantity which the liver and caecum still catabolize with adequate efficiency is around 3–5 mg. Doses larger than this get into the blood circulation, and from there to other organs, as unchanged T-2 toxin (i.e. with unaltered biological activity).

Comparing these values to those measured or known for other species of animals, the rabbit can be considered rather sensitive to T-2 toxin. In our opinion, this may be attributed to coprophagy, by which substantial toxin quantities get back into the digestive tract orally, resulting in prolonged excretion times.

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MACROCYCLIC TRICHOTHECENE TOXINS PRODUCED BY *STACHYBOTRYS ATRA*

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Straw used for feeding different animals and as bedding straw may be infected by *Stachybotrys atra*, a fungus producing highly toxic macrocyclic trichothecenes. These toxins can kill straw-eating animals or predispose animals to fatal secondary diseases, e.g. pasteurellosis of sheep. The amount of the four most important macrocyclic trichothecene stachybotryotoxins (satratoxins G and H, roridin E, verrucarin J) was determined in artificial cultures of 21 *S. atra* strains isolated in Central Europe.

High-pressure liquid chromatography and, after transesterification, gas-liquid chromatography were used for the analysis. All 21 strains produced each toxin but in different amounts. Satratoxin H was consistently present in the greatest amount in the samples. Its LD_{50} value for mice was 1.4 mg/kg intraperitoneally.

Keywords: *Stachybotrys atra*, satratoxins, macrocyclic trichothecene, toxin production

Macrocyclic trichothecene mycotoxins are toxic metabolites produced by fungi, including *Stachybotrys* and *Myrothecium* species (Bamburg and Strong, 1972; Jarvis et al., 1980). These fungi are the causative agents of stachybotryotoxicosis and are known to possess phytopathogenic properties as well. Various reports have been published on macrocyclic trichothecene related mycotoxicosis (stachybotryotoxicosis; Dankó, 1976; Harrach et al., 1983; Schneider et al., 1979; Hintikka, 1977). Such diseases were described in various parts of the world, e. g. in South Africa (Schneider et al., 1979), Finland (Hintikka, 1977), the Soviet Union (Yuskiv, 1968), France (Le Bars et al., 1977), and Hungary (Dankó, 1976; Andrássy et al., 1980; Harrach et al., 1983). Strains isolated in Poland, the German Democratic Republic, Czechoslovakia, Hungary, India and Egypt have been shown to produce stachybotryotoxins. These toxins seem to occur in many countries of the world.

The only available method for the determination of these toxins was described by Stack and Eppley (1980). This method is suitable only for cereals. Since on cereals, however, other fungi tend to overgrow *Stachybotrys atra*, the likelihood of detecting stachybotryotoxins in cereal samples is very low. On other media, e. g. on straw, where only few fungal species are able to grow, it is very probable that *S. atra* becomes the dominant fungus because it can utilize the cellulose present in straw. Straw is widely used as feed and

bedding material for sheep. This may be an explanation for the high incidence of stachybotryotoxicosis in this species. Usually straw of the poorest quality is used for sheep: in most cases the animals consume it without any problem but sometimes a severe disease (stachybotryotoxicosis) develops.

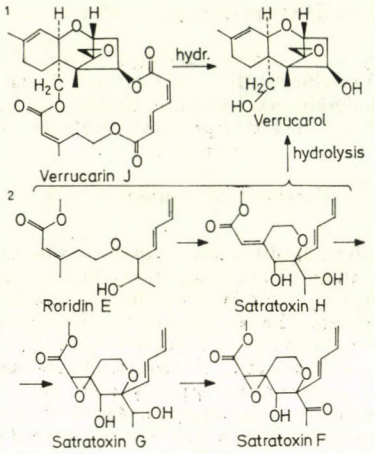


Fig. 1. Chemical structure of the macrocyclic trichothecene toxins

The objective of the present work was to study the toxin production of different *S. atra* strains isolated in Central Europe. The amount of four macrocyclic trichothecene toxins (satratoxins H and G, roridin E and verrucarin J) produced by these isolates was determined (Fig. 1).

Materials and methods

Culture

Stachybotrys atra strains, isolated in various parts of Hungary, Czechoslovakia, and the GDR in connection with stachybotryotoxicosis in animals were cultivated. The isolates were incubated on 100-g portions of sterilized rice, distributed in 2 l Roux bottles plugged with cotton wool, and allowed to harden in horizontal position. The isolates were cultured for 7 days at room temperature, for another 7 days at 4–7 °C, and for 7 days again at room temperature.

Isolation of toxins

The cultures were extracted with 300 ml ethyl acetate for 4 h, then with fresh solvent overnight. Ethyl acetate was evaporated and the oily residue was dissolved in 60 ml acetonitrile and washed with 3×20 ml petroleum ether. The toxin-containing acetonitrile phase was evaporated, the residue was dissolved in 2 ml methanol, and 2 ml water was added to it. This mixture was

passed through a Sep-Pack C_{18} cartridge (Waters Associates Inc., Milford) which was then washed with 2 ml of a methanol : water mixture (1 : 1). The toxins were eluted with 3 ml of methanol : water mixture (8 : 2) from the cartridge which was regenerated with 5 ml methanol : water mixture (1 : 1) before use. The toxin-containing eluent was evaporated to dryness under nitrogen stream at 80 °C. The residue was redissolved in 100 μl methanol.

Thin-layer chromatography (TLC)

Kieselgel-60 10×10 cm HPTLC (Merck, Darmstadt, FRG) plates were used for TLC analysis. The solvent system was chloroform : isopropanol (94 : 6 v/v). Compounds can be detected under UV light (254 nm) or by spraying the plates with 1% 4-(p-nitrobenzyl)-pyridine solution, drying them at 150 °C for 30 min, and spraying with 10% tetraethylene pentamine reagent. The toxins appear as bluish-violet spots against a white background.

High-pressure liquid chromatography (HPLC)

The HPLC separations were performed with a Waters instrument on a 5-μm C_{18} Poligosyl 60D (25 cm×4.6 mm; Macherey-Nagel, Duren, FRG), using the gradient mode of operation. Eluent A was methanol : water 20 : 80 (v/v), and eluent B methanol : water 80 : 20 (v/v). Running time was 20 min.

Peaks eluted with the same retention time as the standard were collected and evaporated to dryness under N_2 stream.

Gas-liquid chromatography

Fifty μl of 0.5 N sodium methoxide reagent was added to the dry residue (Fig. 2). The mixture was placed into a block thermostat and kept there at 60 °C for 15 min. When the reaction was over, 60 μl of methanol, containing

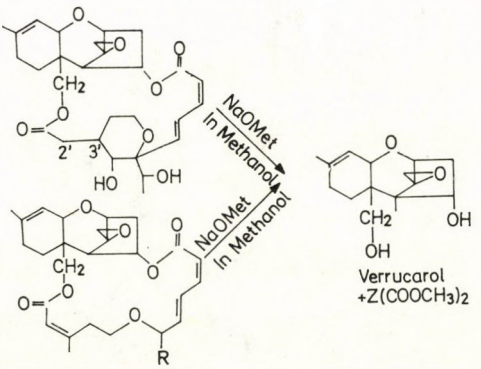


Fig. 2. Transesterification reaction of macrocyclic trichothecene toxins

0.5 N HCl, was added to the mixture to neutralize the base, and the mixture was evaporated to dryness. Subsequently 100 μl of BSTFA reagent was added and the mixture was placed again into a block thermostat at 60 °C for 15 min. To detect the bis-trimethylsilyl ether derivative of verrucarol, a glass capillary wetted with SE 52 stationary phase was used (10 m $\times$ 0.30 mm i.d.). Column temperature was 160–220 °C at a 4 ° C/min rate. The split ratio was 1 : 10.

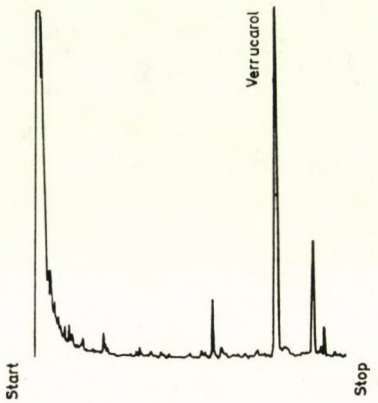


Fig. 3. A typical GLC chromatogram (for conditions, see the text)

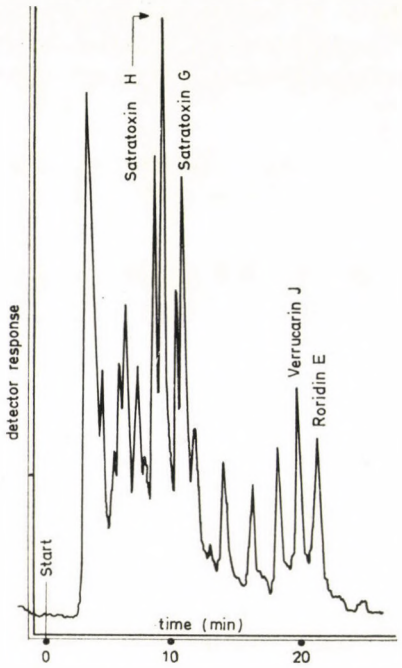


Fig. 4. A typical HPLC chromatogram (for conditions, see the text)

Results

The R_f value of satratoxin G, satratoxin H and verrucarol was 0.53, 0.43 and 0.30, respectively. Fig. 3 shows the gas chromatogram of a sample containing the trichothecene skeleton (verrucarol). Fig. 4 shows the HPLC chromatogram of some samples without transmethylation.

Table I

Retention times and R_f values for the macrocyclic trichothecene toxins tested

Compound	Retention time (min)	R_f value
Satratoxin H	9.2	0.43
Satratoxin G	11.0	0.53
Roridin E	18.8	0.65
Verrucarin J	21.4	0.70
Verrucarol	—	0.30

Table II

Chemical analysis of the *S. atra* strains tested

No. and designation of strains	Satratoxin H	Satratoxin G	Roridin E	Verrucarin J
	(μg/Roux bottle)			
1 Beldvárdgyula	640	280	100	90
2 Budapest I	880	800	170	130
3 Budapest II	540	700	100	60
4 Budapest III	1250	970	260	110
5 Debrecen 23	840	510	50	60
6 Debrecen 1132	850	400	120	120
7 Debrecen 5142	1020	900	210	80
8 Karcag I	650	700	120	60
9 Karcag II	980	870	100	110
10 Miskolc II	160	70	10	10
11 Miskolc III	150	40	0	10
12 Nagytarcsa	950	810	25	70
13 Petrivente	15	0	0	0
14 Perkáta	24	5	0	0
15 Zsarolyán	440	210	140	80
16 MY 276	720	240	180	100
17 MY 93	1610	520	80	30
18 St 1911	510	640	210	—
19 St 1912	740	680	140	—
20 St 1910	210	150	57	—
21 St 1909	340	540	140	—

The sensitivity of the TLC method was 0.3 mg/kg when used for determining the verrucarol content of the sample, and 0.2 mg/kg for both satratoxin G and H.

The sensitivity of the GLC method was 0.05 mg/kg for verrucarol and that of HPLC was 0.01 mg/kg for satratoxins G and H and verrucarin J, and 0.015 mg/kg for roridin E.

The retention times and R_f values of the compounds examined are shown in Table I.

All strains examined in these experiments produced macrocyclic trichothecene toxins. The quantitative results are shown in Table II.

Discussion

In Hungary stachybotryotoxicosis occurs frequently in sheep and other animals consuming large quantities of straw. Straw contaminated with *Stachybotrys* causes substantial economic losses in Central Europe and presumably in other parts of the world as well. All strains of *S. atra* isolated in connection with disease cases were found to produce macrocyclic trichothecene toxins in our experiments, giving an indirect proof of the aetiological involvement of macrocyclic trichothecenes in this disease. Macrocyclic trichothecenes are much more toxic than non-macrocyclic ones (T-2 toxin, diacetoxyscirpenol, etc.). The LD_{50} value of satratoxin H was found to be 1.4 mg/kg, indicating that it is almost four times as toxic as T-2 toxin. It appears from our studies that satratoxins, yielding a total of 0.1–0.2 mg/kg verrucarol, can cause fatal mycotoxicosis in farm animals. Furthermore, stachybotryotoxins frequently predispose animals to fatal secondary diseases. As a result of the impairing effect exerted by satratoxin on the immune system, *Pasteurella haemolytica* biotype T strains are activated and multiply in the tonsils earlier. This frequently leads to mass fatal disease in adult sheep. The condition is usually diagnosed as acute pasteurellosis facilitated by feeding and zoohygienic problems (Hajtós et al., 1983).

The veterinary pathologist should consult a mycotoxin analytical laboratory in every case of pasteurellosis when sheep are losing their fleece, when lesions are seen in their oral cavity and digestive tract, when *P. haemolytica* cannot be isolated from all carcases, and when antibiotics are not effective against the disease. Treatment should include removal of the satratoxin-contaminated straw and the use of antibiotics effective against activated pasteurellosis.

Acknowledgement

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ULTRASTRUCTURE OF MICROGAMETOGENESIS OF *EIMERIA STIEDAI* IN RABBITS *(ORYCTOLAGUS CUNICULUS)*

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Ultrastructural aspects of microgametogenesis of *Eimeria stiedai* in the bile duct epithelium were studied in experimentally infected rabbits by transmission and scanning electron microscopy after cryo-fracture. Developing microgamonts had many fissures in the cytoplasm. Extensive invaginations produced cytomeres in the mature stages around the boundaries of which microgametes differentiated. Each microgamete had two flagella and four supportive internal microtubules.

Keywords: *Eimeria stiedai*, microgametogenesis, ultrastructure, rabbit

Fine structural studies on microgametogenesis of *Eimeria* in rabbits have been mainly confined to the intestinal species. This process in *E. perforans* was briefly reported by Scholtyseck and Spiecker (1964), Hammond et al. (1967), Scholtyseck et al. (1972), and Streun et al. (1979), and more extensively by Scholtyseck in 1965. Microgametogenesis in *E. intestinalis* has been recorded by Cheissin (1965) and Snigirevskaya (1969), and in *E. magna* by Cheissin (1965), Snigirevskaya (1969), and Speer and Danforth (1976).

Apart from *E. hiepei* reported by Grafner et al. (1967) in the bile ducts of a mink, *E. stiedai* is unique amongst the mammalian coccidia in that it develops in the bile duct epithelium of the rabbit, *Oryctolagus cuniculus*, the hare, *Lepus europaeus* (Varga, 1976*a*, 1976*b*), and possibly in other Leporidae (Pellérdy, 1974). Apart from a brief description of the early microgamont of this parasite (Hammond et al., 1967) there are to the best of our knowledge no other published data relating to the microgamont or its development for this species.

Scanning electron microscopy has been used extensively in the study of coccidial infections. Most of the information obtained by this technique has been more relevant to the pathology of the infection than to parasite structure itself. The reason for this is that when conventional techniques are used to examine intestinal surfaces, the parasites will be revealed only if the host cells are ruptured as a result of pathological events or as a preparative artifact. For example, it was possible to use scanning electron microscopy to examine microgametogenesis of *E. maxima* (Ball et al., 1981) because surface damage

occurred to such an extent that many organisms were revealed. More recently, however, we have examined cryo-fractured tissue containing *E. acervulina* and used this methodology along with transmission electron microscopy to report macrogametogony and oocyst wall formation for this species (Pittilo and Ball, 1984). This technique exposes internal surfaces and allows fields to be examined that would not normally be accessible to the scanning electron microscope.

In this paper we employ similar methods to examine microgametogenesis of *E. stiedai* and to investigate the possibility of structural and developmental differences between this species and other eimerian parasites, particularly those in the rabbit.

Materials and methods

Three specific pathogen free New Zealand white female rabbits of 2 to 2.5 kg body weight were each inoculated orally with 1×10^4 sporulated oocysts of the strain *E. stiedai* originally supplied by Dr M. E. Rose. Liver tissue was collected on days 19, 21 and 23 after inoculation and prepared by the methods described previously (Pittilo and Ball, 1979, 1984).

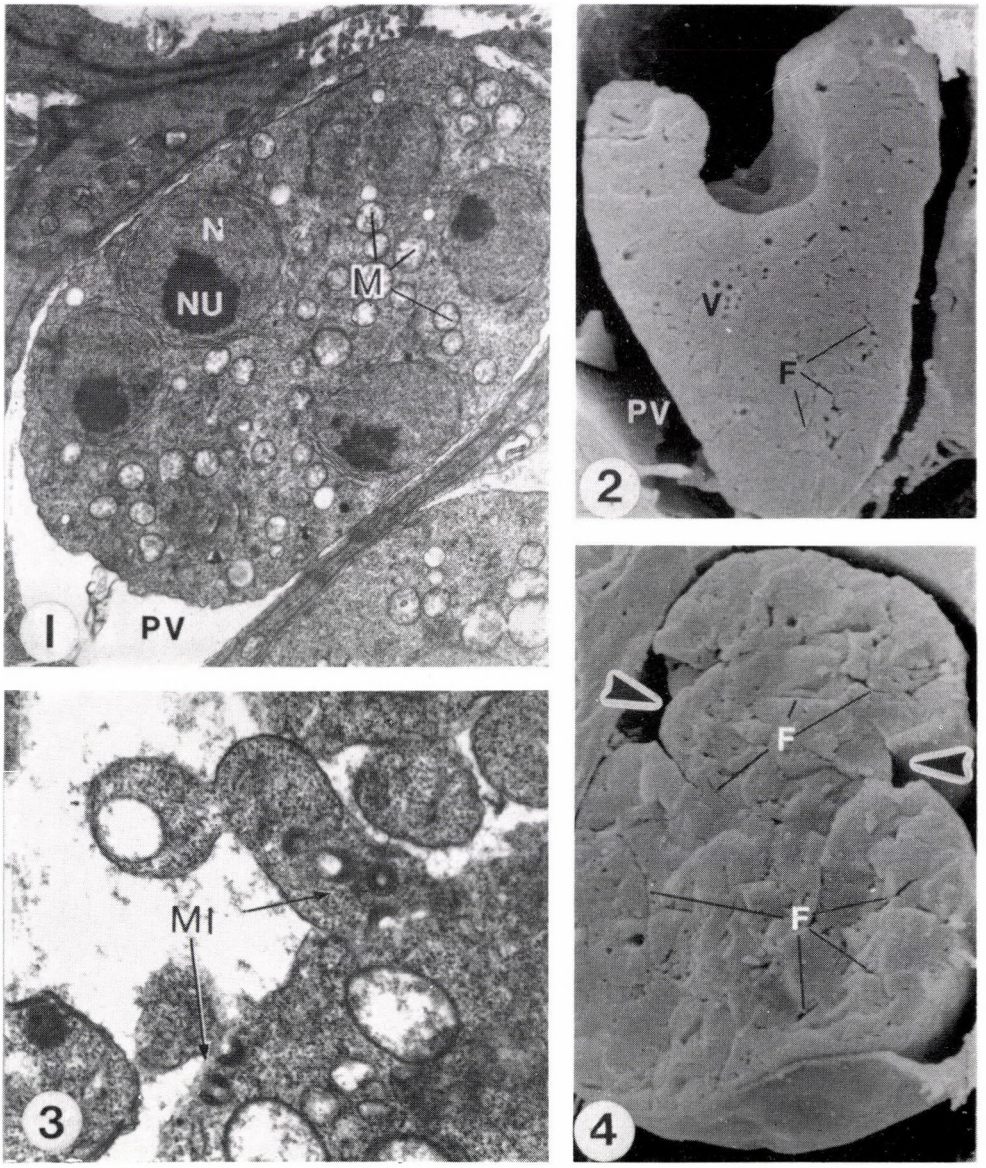
Results

The surface morphology of non-fractured infected bile duct epithelium showed an irregular surface with many of the cells protruding. In areas of severe damage it was possible to observe the surface structure of exposed parasites. The fractured surfaces showed a heavily parasitised epithelium in which microgamonts at all stages of development could be seen at the three different times examined.

The young microgamont developed in a parasitophorous vacuole limited by a host membrane devoid of intravacuolar folds (Figs 1 and 2). The early stages had nuclei, with prominent nucleoli, distributed throughout an undivided cytoplasm which contained many mitochondria (Fig. 2). Micropores in the parasite membrane were evident (Fig. 3).

As the microgamont increased in size numerous euchromatic nuclei were present and the cytoplasm contained many vesicles (Fig. 5). A notable feature at this stage, and throughout subsequent development, was the presence of clefts or fissures in the parasite cytoplasm (Figs 4 and 5). Paired centrioles were evident in the cytoplasm or positioned between the nuclei and the microgamont surface (Fig. 5).

During further development the microgamont became divided into cytomeres (Figs 6–9) resulting in section in the appearance of islands of cytoplasm



Figs 1–4. Young microgamonts of *Eimeria stiedai*

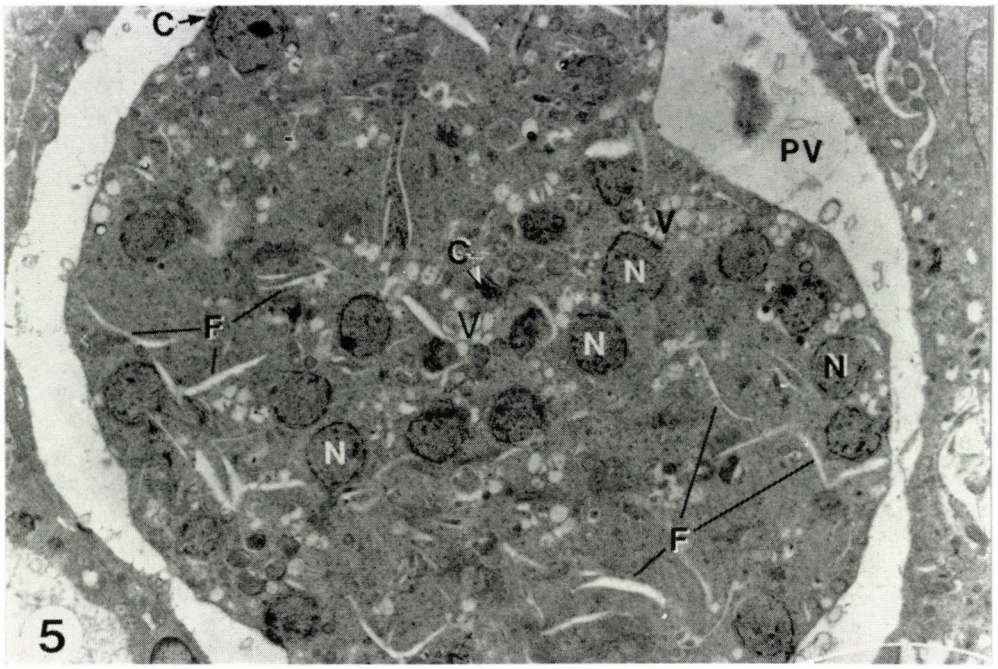
Fig. 1. Transmission electron micrograph (TEM) of young microgamont within parasitophorous vacoule. Nuclei have large nucleoli and many mitochondria are present in the cytoplasm. ×7,000

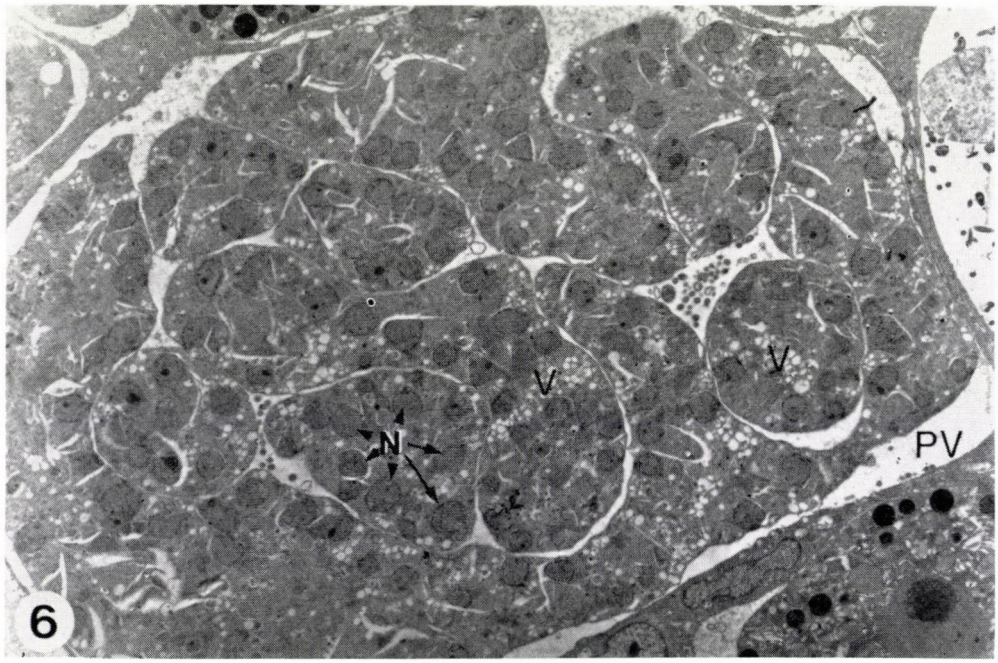
Fig. 2. Scanning electron micrograph (SEM) of cryo-fractured tissue showing a young microgamont. Vesicles and small fissures are present. ×6,500

Fig. 3. Micropores in the young microgamont. TEM, ×23,400

Fig. 4. SEM of cryo-fractured tissue showing a growing microgamont with fissures and invaginations (arrow heads) of the surface. ×3,800

Abbreviations: C = centriole; F = fissure; FL = flagellum; M = mitochondrion; MI = micropore; N = nucleus; NM = nucleus of microgamete; NU = nucleolus; PF = paired flagella; PV = parasitophorous vacuole; V = vesicle

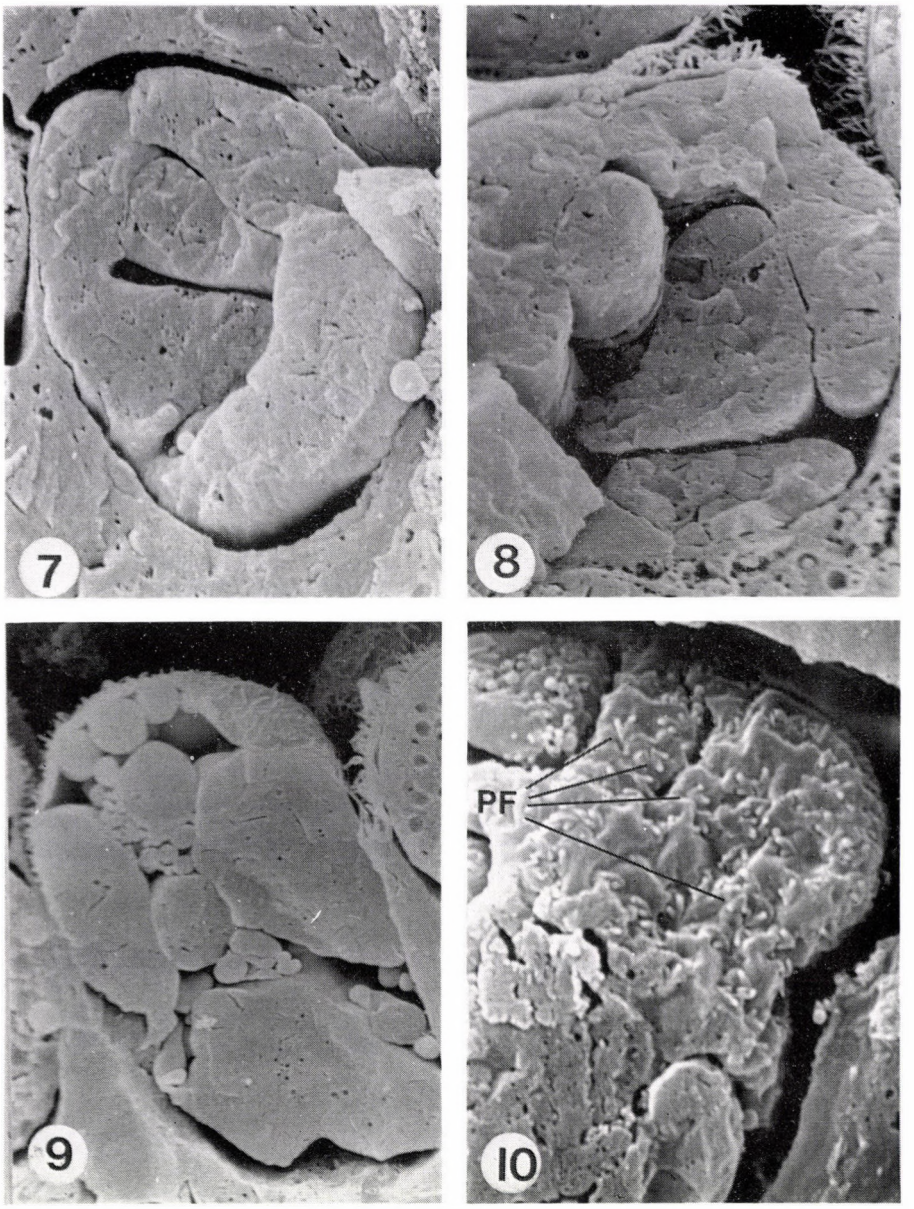




Figs 5 and 6. TEM of growing microgamonts

Fig. 5. Similar stage to Fig. 4. Nuclei have dispersed heterochromatin. Centrioles are evident in places. ×6,100

Fig. 6. Large microgamont showing cytoplasm divided into cytomeres. Vesicles and fissures are prominent and nuclei tend to be in peripheral positions in the cytoplasmic masses. ×2,800



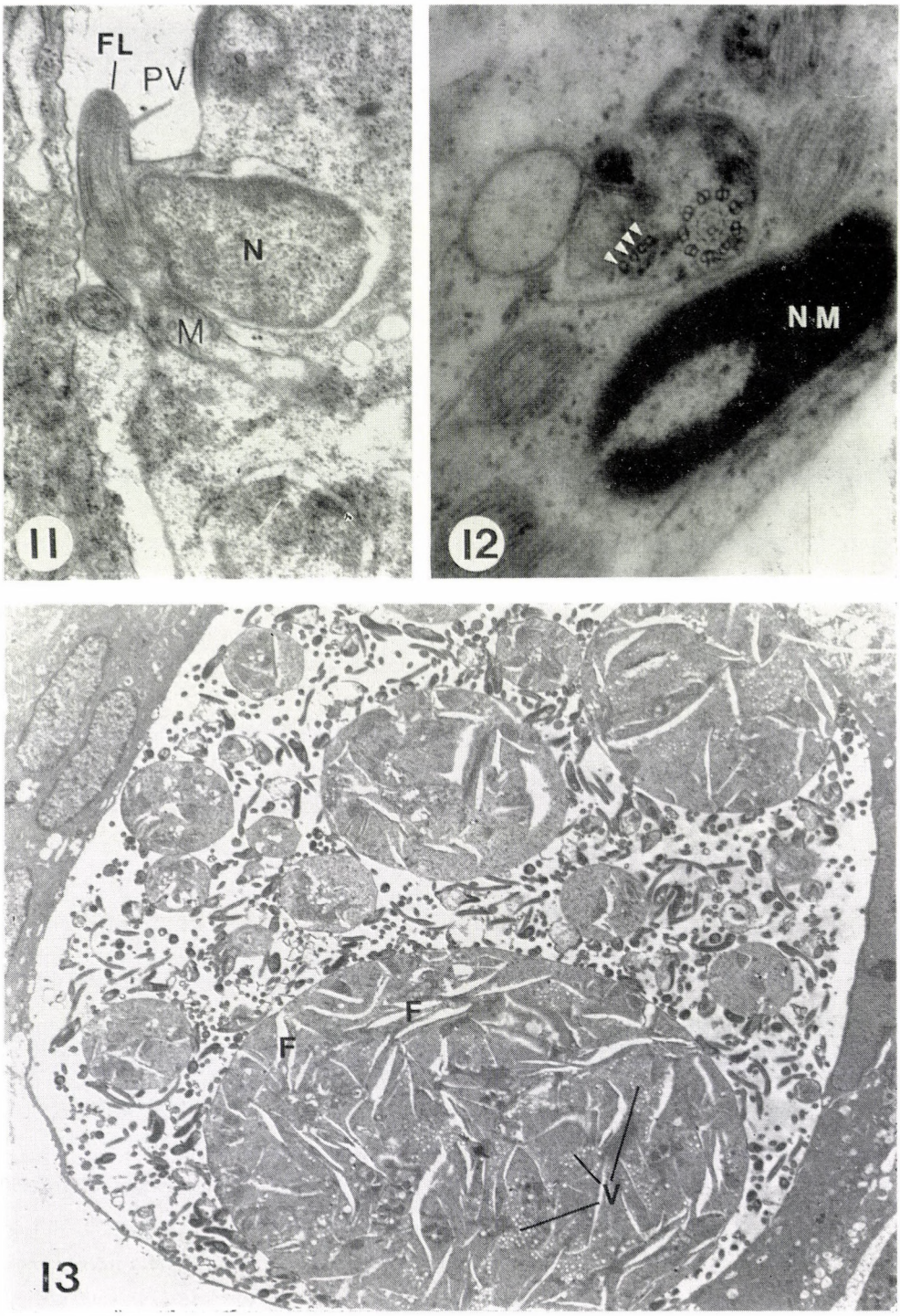
Figs 7–10. SEM of maturing microgamonts

Fig. 7. Microgamont showing deep invaginations and beginning of cytomere formation. ×3,400

Fig. 8. Large cytomeres are evident. ×3,800

Fig. 9. Large and numerous small cytomeres. ×2,700

Fig. 10. Paired flagella emerging from the surface of microgamont during early microgamete formation. ×11,200



Figs 11–13. TEM of differentiating microgamonts

Fig. 11. Emergent flagellum of a microgamete shows close association of nucleus and mitochondrion. ×23,600

Fig. 12. Developing microgamete showing 4 supportive microtubules (arrow heads). ×50,000

Fig. 13. Mature microgamont with large numbers of microgametes within the parasitophorous vacuole. The residual cytoplasm (the cytomeres) contain fissures and vesicles. ×2,800

each with several nuclei which tended to lie along the periphery (Fig. 6). Many of the clefts appeared to be in close association with the nuclei (Fig. 6).

As flagella elongated from the surface of the microgamont (Figs 10 and 11), the nuclei were undergoing differentiation in which the heterochromatic part became more distinguishable. At this stage, each nucleus was associated with two flagella and a mitochondrion (Fig. 11). The maturing microgamont occupied the entire greatly enlarged bile duct cell.

Microgametes were released into the parasitophorous vacuole (Fig. 13) and each had two free flagella (Fig. 10). Large amounts of residual cytoplasm remained in which many vesicles and fissures were present (Fig. 13). Within the developing microgamete four microtubules were situated between a basal body and the mitochondrion (Fig. 12).

At days 21 and 23 after inoculation microgamonts were at various stages of development in any one bile duct section but the formation of microgametes in any one parasite was synchronous.

Discussion

The number of species of *Eimeria* examined by electron microscopy is sufficient to allow general conclusions to be drawn concerning morphogenesis. The basic pattern of growth of the microgamont during which there is nuclear division, followed by a phase when microgametes are differentiated was first propounded by Scholtyseck et al. (1972), and has been substantiated by subsequent studies on microgametogenesis. *E. stiedai* does not deviate from this. However, it was notable that the surface area of the microgamont of *E. stiedai* was increased by deep invaginations of the pellicle to form cytomeres in section, along the outer surface of which microgametes developed. In general, the relatively small sized microgamonts of several species of coccidia (see Scholtyseck et al., 1977) are not subdivided, but large microgamonts often developed invaginations which provide additional surface area for the production of large numbers of microgametes. Therefore, *E. stiedai* is similar to *E. magna* as depicted by Cheissin (1965) in the intestinal cells of the rabbit.

Presumably microgamonts obtain nutrients by osmotrophy and the use of micropores which were abundant in the young stages of *E. stiedai*. Intensive metabolism of the early stages of development is reflected in the large number of mitochondria present in the cytoplasm. Accompanying the growth of the microgamonts the number of vesicles increased and many remained in the residual cytoplasm after microgamete formation. These may have contained lipid. No polysaccharide granules were observed.

Reports differ on the distribution of chromatin within the nucleus which is to be incorporated into the microgamete and whether or not the whole

nucleus is involved. Cheissin (1965) suggested that a whole nucleus was employed in the formation of a microgamont of *E. intestinalis* and *E. magna* in the rabbit, as was reported for *E. perforans* (Scholtyseck et al., 1972). The present study suggests that this also occurs in *E. stiedai*. By contrast, only the condensed chromatin portion was shown to be incorporated in the microgametes of *E. intestinalis* (Snigirevskaya, 1969) and *E. magna* (Snigirevskaya, 1969; Speer and Danforth, 1976).

As pointed out by Scholtyseck et al. (1977), it is difficult to accurately ascertain the number of flagella present in all coccidial microgametes, and they list references supporting the conclusion that those species having two or three flagella are about equal in number. All eimerian microgametes have two free flagella as shown here for *E. stiedai* which does not possess a third, but does have a row of four microtubules originating in the anterior end.

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ELECTRON MICROSCOPY OF *EIMERIA BAKUENSIS* (SYN. *E. OVINA*): MACROGAMETOGONY AND OOCYST WALL FORMATION

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Macrogametes were first identified by the appearance of wall-forming bodies of type 2 (WFB II). Maturation resulted in the formation of wall-forming bodies of type 1 (WFB I), and the accumulation of food reserves in the form of polysaccharide granules and lipid. The means by which the parasite obtains nutrients during development is discussed. Oocyst wall formation occurred by a typical eimerian pattern involving the WFB I and WFB II. Membranes were shed from the developing oocyst during this process and the outer layer consisted of an osmiophilic and a granular region.

Keywords: *Eimeria bakuensis*, macrogamete, oocyst, ultrastructure

Eimeria bakuensis Musaev, 1970 (syn. *E. ovina)* has recently been shown to be the correct name for the ovine parasite which is the counterpart of *E. arloingi* in the goat (Norton, 1986). Lotze (1953) published a description of the life cycle of this parasite, then known as *E. arloingi*.

The purpose of this paper is to report the ultrastructure of the macrogamete and oocyst wall formation in this species. In view of the large literature on these developmental stages in coccidia, we have restricted the discussion to novel findings only.

Materials and methods

Material was obtained from a 9-week-old lamb that had been raised coccidia-free. At 5 weeks of age it had been inoculated with *E. parva* to obtain a culture. At 9 weeks of age it showed transient adventitious infection with *E. ovinoidalis*. At this time it was inoculated with 10^5s porulated oocysts of *E. bakuensis*. No oocysts of any species were produced between day 2 and day 21 after the *E. bakuensis* inoculation. Between day 21 and day 24, when it was killed, 5.9×10^9 oocysts of *E. bakuensis* were produced. Tissue samples from an oocyst patch in the jejunum were taken under deep pentobarbitone anaesthesia on day 24.

The tissue was collected into 2.5% glutaraldehyde in 0.1 M cacodylate buffer at pH 7.2. Following primary fixation the tissues were washed with fresh buffer and post-fixed in 1% osmium tetroxide for 30 min. Using standard techniques, the tissues were dehydrated through alcohol, transferred to a solution of propylene oxide then embedded in Araldite.

Thin sections were cut on a Reichert OMU4 ultramicrotome and after staining with lead citrate examined in Jeol 100S and Philips 301 transmission electron microscopes.

Results

Macrogametes could first be identified by the appearance of wall-forming bodies of type two (WFB II) (Fig. 1). These were first identified as round structures of medium but not uniform electron density that were surrounded by granular endoplasmic reticulum (Fig. 1). Wall-forming bodies of type one (WFB I) were frequently only apparent as small highly osmiophilic granules even when numerous well-formed WFB II could be identified (Fig. 1).

Further development of the WFB II was accompanied by little morphological change other than an increase in size and a tendency to become more sponge-like in appearance and have a preference to locate in a ring interior to the rapidly developing WFB I which were now situated immediately below the limiting membranes of the parasite (Figs 2 and 3). Occasionally WFB II with altered structure were apparent with electron pale islands present within the WFB II material (Figs 4 and 5). Also, in places the WFB II material was so closely applied to the granular endoplasmic reticulum that the usual space seen was occluded (Fig. 4).

Polysaccharide granules were sparse in young macrogametes (Fig. 1) but were abundant in mature macrogametes (Figs 2, 4 and 5). The electron density of the polysaccharide was variable (compare Fig. 2 with Fig. 5). Usually large amounts of lipid were evident in all but the youngest macrogametes and were mainly contained within the canaliculi (Fig. 4).

The parasitophorous vacuole was of variable width and often contained granular material (Fig. 2). Intravacuolar folds were found within the parasitophorous vacuole (Figs 2 and 3). Intravacuolar tubules were only observed within the parasitophorous vacuole of one macrogamont, where a portion of the tubules was seen (Fig. 3). The parasite was limited by two membranes with interrupted membrane portions present below the inner membrane (Fig. 3). Micropores were rarely seen.

A prominent nucleolus was present in a number of sections through the parasite nucleus (Fig. 6). Mitochondria, granular endoplasmic reticulum and free ribosomes were abundant throughout the cytoplasm (Fig. 3).

Formation of the outer layer of the oocyst wall results from a transfer of material of the WFB I to the margin of the parasite to form what is initially an irregular osmiophilic layer (Fig. 7). This layer became more regular in width but was not of uniform electron-density (Figs 8 and 9) until after the inner layer of the oocyst wall had been fully formed. At this stage the major part of the outer layer of the oocyst wall was again osmiophilic (Fig. 10).

When the bulk of the WFB I material had been transferred to the perimeter of the parasite, only WFB II remained within the developing oocyst (Figs 6, 7, 8 and 9). At this stage, the WFB II were very sponge-like in appearance and existed as islands within electron pale areas (Figs 6 and 9). Formation of the inner layer of the oocyst wall was associated with the disappearance of WFB II from the oocyst cytoplasm (Fig. 10).

Fully formed oocysts were poorly infiltrated with resin which rendered them difficult to examine (Fig. 10). There was evidence to indicate that two membranes are shed from the oocyst after oocyst wall formation (Figs 8 and 10). The outer layer of the oocyst when fully developed is highly osmiophilic with some granular material on the surface (Fig. 10).

Discussion

The structure of the developing macrogametes of protozoa belonging to the genus *Eimeria* show considerable uniformity and this has been reviewed by Scholtyseck et al. (1971) and Scholtyseck (1973). In general, *E. bakuensis* conformed with the usual developmental processes of macrogametogony, although some novel aspects were noted.

In this study macrogametes were first identified by the appearance of WFB II. It was usual to see stages with morphologically mature WFB II but with the WFB I only recognisable as small osmiophilic granules. This is consistent with the WFB II being formed in advance of the WFB I (Scholtyseck, 1973).

WFB II structure was unusual in some places in that electron pale areas existed within some WFB II. It is possible that there are a number of biochemical paths by which wall-forming material is accumulated and intermediate metabolic products may present a varying morphology.

The accumulation of polysaccharide granules and lipid during development of the macrogamete is normal for eimerian macrogametes (Scholtyseck et al., 1971; Scholtyseck, 1973). The means by which macrogametes of *E. bakuensis* obtain nutrients is unclear but it is probable that close apposition between the parasite and the host cell membrane of the parasitophorous vacuole, micropores and intravacuolar tubules are all involved to varying extents. We could easily have reported intravacuolar tubules as being absent in this

species as they were only observed once. If the nutritional hypothesis is correct for intravacuolar tubules (Hammond et al., 1967; Scholtyseck et al., 1971; Scholtyseck, 1973; Michael, 1975), it is possible that they may be required for nutrition only when other means are insufficient. Although structural connections have been reported between the host cell, intravacuolar tubules and macrogamete surface (Scholtyseck et al., 1971; Michael, 1975; Varghese, 1975; Pittilo and Ball, 1979) for a number of species, information is lacking as to the means of formation of the tubules or whether or not they can be readily assembled and disassembled as can occur with microtubules. It may be that species reported to have no intravacuolar tubules including *E. ferrisi* (Chobotar et al., 1975), *E. stiedai* (Scholtyseck et al., 1966; Pittilo et al., 1980), and the related coccidia *Isospora felis* and *I. rivolta* (Pelster, 1973) and *Toxoplasma gondii* (Pelster and Piekarski, 1972; Ferguson et al., 1975) may eventually be shown in certain circumstances to be capable of forming the tubules.

The parasitophorous vacuole contained granular material that was often of similar electron density to the host cell cytoplasm. It has been postulated that the intravacuolar folds become detached from the host cell membrane forming particulate material within the parasitophorous vacuole that may be taken into the parasite (Scholtyseck, 1973). This could account for the origin of the granular material in the case of *E. bakuensis*. An alternative explanation may be that it represents waste material from the parasite. There are considerable gaps in our knowledge as to how macrogametes obtain their nutrients and dispose of waste products and all attempts to explain them must at the present time remain speculative.

The complete process of oocyst wall formation was not observed although evidence is presented to indicate that for *E. bakuensis* it does not differ other than in detail from that considered to be normal for coccidia (Scholtyseck et al., 1971; Scholtyseck, 1973). The existence of membranes surrounding the developing oocyst is well recognised in a number of coccidia and this has been discussed in some detail in relation to the available literature for *E. brunetti* (Ferguson et al., 1977), *E. maxima* (Pittilo and Ball, 1980), and *E. acervulina* (Pittilo and Ball, 1984). In the case of *E. bakuensis*, they almost certainly arise from the original parasite limiting membranes and the surrounding membrane of the WFB I.

E. bakuensis is unusual in that the WFB II are found within large electron-pale areas during formation of the oocyst wall. A similar phenomenon occurred with the WFB I in the formation of the oocyst wall of *E. acervulina* (Pittilo and Ball, 1984). In the case of *E. bakuensis* it is possible that some of the electron-pale material is lipid derived from the large amounts that were present within the mature macrogamete. The mechanism by which WFB II material is transferred from the cisternae of the granular endoplasmic reticulum to the exterior of the parasite is unclear. Interpretation of this process

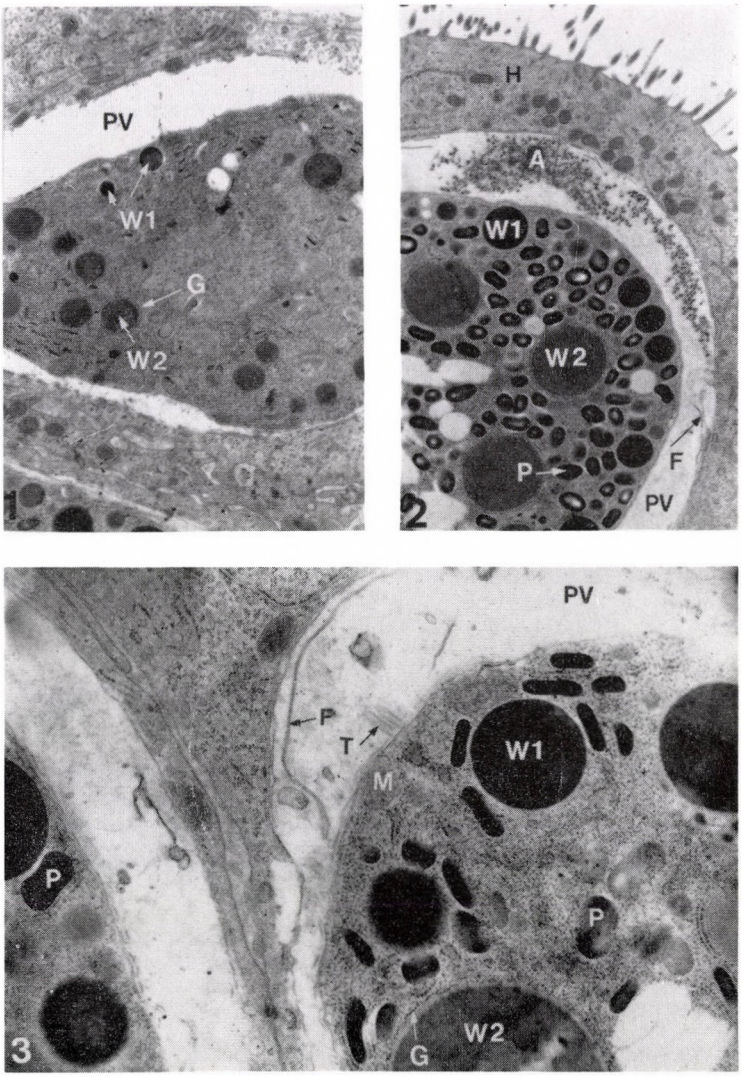


Fig. 1. A young macrogamete of *E. bakuensis.* Wall forming bodies of type two (W2) surrounded by granular endoplasmic reticulum (G) are easily recognisable, but at this stage wall-forming bodies of type one (W1) are only present as small osmiophilic structures within the cytoplasm. The parasitophorous vacuole (PV) is electron pale. ×8,100

Fig. 2. Part of a mature macrogamete of *E. bakuensis.* Within the parasitophorous vacuole amorphous material (A) and intravacuolar folds (F) can be seen. Many polysaccharide granules are present within the cytoplasm of the macrogamete. This parasite is found just below the brush border of the host cell (H). ×6,700

Fig. 3. A portion of two mature macrogametes of *E. bakuensis.* Within the parasitophorous vacuole of one of the stages, intravacuolar folds and intravacuolar tubules (T) can be seen. The parasites are limited by two complete membranes below which some interrupted membrane portions are found. The WFB II (W2) are surrounded by granular endoplasmic reticulum. ×19,200

Abbreviations used on figures

A: amorphous material; AR: artefact; C: canaliculi; F: intravacuolar fold; G: granular endoplasmic reticulum; H: host cell; I: inner layer of oocyst wall; M: mitochondrion; N: nucleus; Nu: nucleolus; O: outer layer of oocyst wall; P: polysaccharide granule; PV: parasitophorous vacuole; S: electron pale space; T: intravacuolar tubule; W1: wall-forming body of type one; W2: wall-forming body of type two

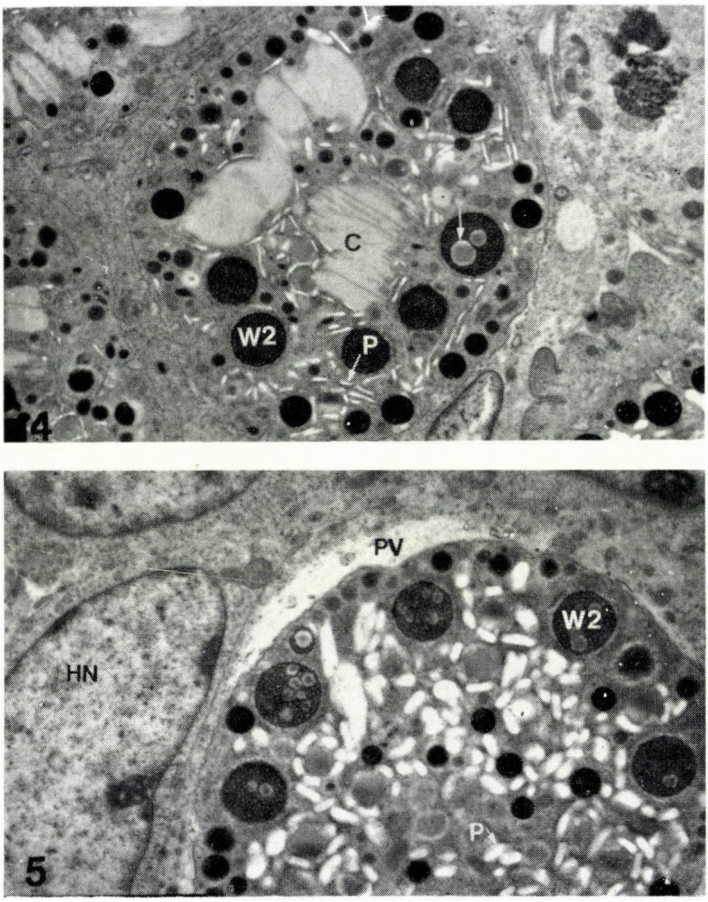


Fig. 4. Part of a mature macrogamete of *E. bakuensis.* A pale island within a WFB II is arrowed. The polysaccharide granules appear electron pale. The parasitophorous vacuole is difficult to discern due to its having similar electron density to the host cell cytoplasm and because the parasite is in places closely applied to the limiting membrane of the parasitophorous vacuole. Canaliculi (C) containing lipid are shown. ×6,600

Fig. 5. Part of a mature macrogamete of *E. bakuensis.* The WFB II have pale islands within their material. The polysaccharide granules are electron pale. ×8,300

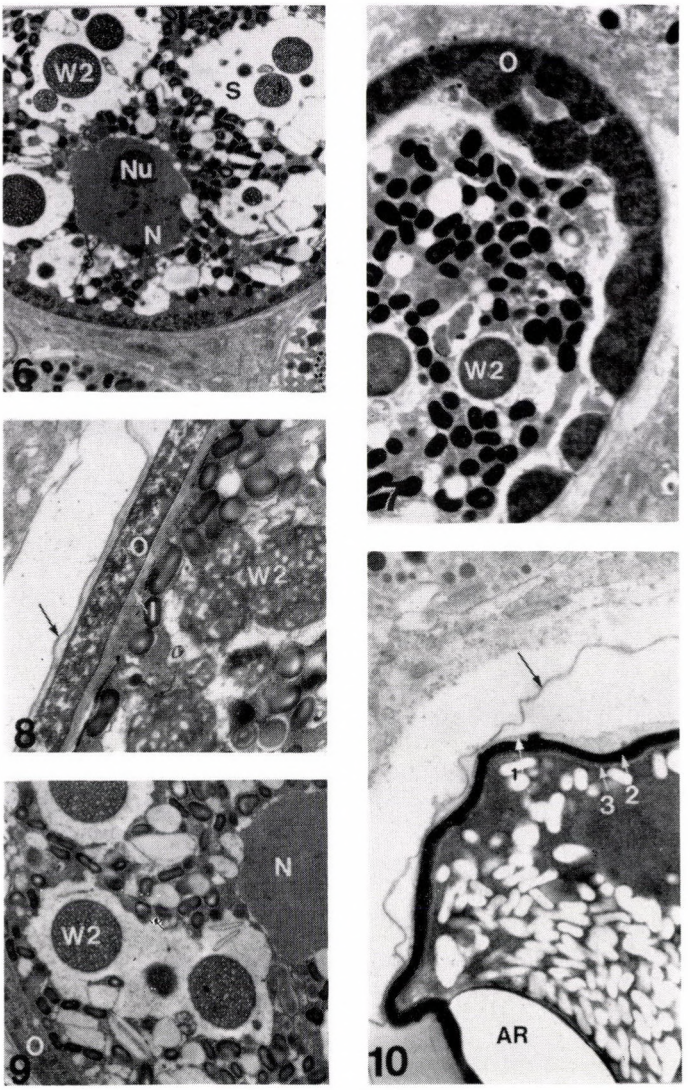


Fig. 6. An oocyst of *E. bakuensis.* The WFB I have disappeared from the cytoplasm. Within the cytoplasm a prominent nucleus (N) with nucleolus (Nu), WFB II surrounded by electron pale spaces (S), and polysaccharide granules can be discerned. ×4,200

Fig. 7. An oocyst of *E. bakuensis.* The outer layer of the oocyst wall (0) is formed by the fusion of WFB I at the perimeter of the parasite. At this stage the outer layer is irregular and osmiophilic. WFB II can be seen within the cell. ×6,700

Fig. 8. An oocyst of *E. bakuensis.* The outer layer of the oocyst wall is of regular width but of variable electron density. A thin granular layer separates this layer from a surface membrane (arrowed). Although WFB II can be seen within the cell, formation of the inner layer (I) has commenced. ×11,100

Fig. 9. An oocyst of *E. bakuensis* at a similar stage of development to that seen in Fig. 6. Fine amorphous material appears to be within the electron pale areas surrounding the WFB II. ×6,700

Fig. 10. Part of an oocyst of *E. bakuensis* is shown in which the oocyst wall is fully formed. After formation of the oocyst wall, the oocyst cannot be satisfactorily prepared for electron microscopy using conventional techniques and a distorted appearance with artefacts (AR) such as tearing away of the cytoplasm frequently occurs. Arrows mark the granular part of the outer wall (1), the osmiophilic part of the outer wall (2) and the inner layer of the oocyst wall (3). The membrane released from the oocyst wall shown in Fig. 8 can again be seen (arrow). Electron pale polysaccharide granules fill the cytoplasm. ×7,400

morphologically is hampered by the difficulty in preparing thin sections of oocysts for electron microscopy that results after even partial formation of the oocyst wall has taken place. Alternation of the outer layer of the oocyst wall resulting in its consisting of osmiophilic and granular parts has been reported for a number of *Eimeria* species (see Pittilo and Ball, 1984).

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EFFICACY OF FUMAGILLIN AGAINST *MYXIDIUM GIARDI* CÉPÉDE, 1906 INFECTION OF THE EUROPEAN EEL *(ANGUILLA ANGUILLA)*: NEW OBSERVATIONS ON MYXIDIOSIS OF IMPORTED GLASS EELS

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When checked on importation in Hungary, glass eel stages of the European eel *(Anguilla anguilla)* caught at the Atlantic coast proved free from parasitic infection. After a few weeks, however, the plasmodia and cysts of *Myxidium giardi* were demonstrable in both the eels kept in a laboratory and those reared at an eel farm, indicating that the fish had been infected by the parasite latently already upon arrival in Hungary.

To prevent the development of infection, the elvers were fed fumagillin mixed in the feed at a dosage of 0.1% or 1.0%. Continuous feeding of the fumagillin-containing diet prevented the development of spores in the yet asymptomatic fish stocks, and reduced the intensity of infection in eels showing an advanced stage of infection.

Keywords: *Myxidium giardi*, *Anguilla anguilla*, renal parasite, therapy, fumagillin, biology

Until quite recently, no efficacious drug has been known to exist against myxosporeans, parasites that cause significant diseases in fishes. Finally in 1987 Molnár et al. (1987) reported that, when mixed in the feed in a concentration of 0.1%, Fumagillin DCH (CHINOIN Pharmaceutical and Chemical Works Ltd., Budapest, Hungary) prevented the spore formation of the common carp parasite *Sphaerospora renicola*. Earlier fumagillin had only been used against microsporeans, parasites taxonomically distant from myxosporeans. Katznelson and Jamieson (1952) were the first to use this antibiotic against nosema disease of the honey bee. In fish, Kano and Fukui (1982) reported successful use of fumagillin against microsporeosis of the Japanese eel *(Anguilla japonica)* caused by *Pleistophora anguillarum*.

Myxidium giardi infection of the eel is one of the most significant and best known eel parasitoses. Data on its pathology were reported by Copland (1983), Crane and Eversole (1980), Ghittino et al. (1974), Landsberg (1983), and Ventura and Paperna (1984). Many years' experience suggests that in glass eels imported to Hungary *M. giardi* infection appears within a few weeks and gives rise to substantial pathological lesions.

The favourable effect of fumagillin against renal sphaerosporosis of the common carp prompted us to test the efficacy of this antibiotic against eel

myxidiosis. The efficacy of Fumagillin DCH against myxidiosis is reported in this paper, along with observations made on *Myxidium* infection of imported glass eels.

Materials and methods

The experiments were conducted under laboratory conditions, in 401 aquaria, and at a warm-water eel farm in 1987. In the laboratory experiments we used imported glass eels that had not fed in Hungary before, and pigmented eels that had been reared at a Hungarian eel farm for 2–3 months. The glass eels had been caught in river mouths at the Atlantic coast of France. Two batches of eels were used: the first transport arrived in Hungary on 20 February 1987 and the second one month later, on 21 March 1987. Upon arrival the glass eels were just starting to feed. In the adaptation period lasting about 3 weeks prior to starting the laboratory experiment they received cut-up tubifex. Subsequently a gradual transition was made to the medicated or the control diet. The control diet used in the experiments was the special floating eel feed manufactured by Tagger and Co. GmbH (Graz, Austria). This was supplemented with Fumagillin DCH to obtain the medicated diet. The antibiotic fumagillin is produced by the fungus *Aspergillus fumigatus* and manufactured as dicyclohexylamine salt (DCH) by CHINOIN Pharmaceutical and Chemical Works Ltd. (Budapest, Hungary). Its chemical name is 2, 4, 6, 8-decatetraenedioic acid (4-(1,2-epoxy-1,5-dimethyl-4-hexenyl)-5-methoxy-1-oxaspiro (2,5)oct-6-yl) monoester. The eels were assigned to groups and fed a medicated diet containing 0.1 or 1.0% Fumagillin DCH, or the control diet, until killed. The treatment period varied between 19 and 61 days. The eels assigned to the different experimental groups varied in number (Table I).

At the end of the experiment the eels were anaesthetized and dissected. Squash preparations made from the kidney were examined for the presence of *M. giardi* plasmodia and spores by light microscopy, under a coverslip. Although *Myxidium* cysts may occur in the gills and in other organs as well, their number is negligible as compared to that found in the kidneys. On the basis of the spore counts, the intensity of infection was rated as one to three crosses (see Tables I and II), for lack of a more accurate method. Unfortunately, undoubtedly parasite-free eels cannot be used, since this fish species cannot be cultured artificially (only few promising attempts have been made at its artificial culture).

The farm-pond trials were conducted at the eel farm of the BHG on two occasions in 1987. In the first experiment eels from the first transport were fed a diet enabling them to acclimatize, containing tubifex, then tubifex plus fish pulp, for two weeks. Subsequently the eels were assigned to two groups. Eels of the control group received a normal, unmedicated eel diet, whereas

those of the experimental group were fed the medicated diet containing 0.1% Fumagillin DCH four times daily, for 11 days. In the second experiment, lasting 76 days, eels from the second transport batch were used after the adaptation period. The eels were fed the medicated diet four times a day over the first 9 days of the experiment. Subsequently, over the remaining 67 days the fish were fed the medicated diet once and the control one three times a day. The first experiment was evaluated after 11 days, and the second one six times during the 76-day medication period by random sampling. In the field trial each experimental group consisted of eels from the same tank.

Results

Parasitological examination of the glass eels

Upon arrival in Hungary, eels of both transport batches, originating directly from the Atlantic coast, appeared to be parasite-free.

Twenty-five and 38 days after the first importation (i.e. at the beginning of the experiment, after the adaptation period), the untreated eels reared in aquaria proved free from parasites. Forty-seven days after importation, however, *Myxidium* infection became apparent and reached a prevalence of 30%. Fifty-six and 77 days after arrival the prevalence of infection reached 80 and 100%, respectively. The intensity of infection was low (+) at all times studied.

Twenty days after the second importation (i. e. at the beginning of the experiment, after the adaptation period) the untreated eels reared in aquaria already exhibited an infection of low prevalence and intensity. Forty-nine days after importation (i.e. at the end of the experiment) the prevalence of infection was 85% and its intensity was moderate (++).

Medication trials against Myxidium giardi in eels kept under laboratory conditions (Table I)

In the first experiment the eels received the medicated diet for 22 days. One out of the 17 fish fed 0.1% Fumagillin DCH showed infection of low intensity, whereas all 18 fish fed the medicated diet containing 1.0% Fumagillin DCH remained parasite-free. In the control group an infection of low intensity and 30% prevalence (4 out of 12) was found. (In the first experiment latently infected eels of the first transport batch were used.)

The second experiment was evaluated in two parts. On day 19 of medication all 8 eels fed 1.0% Fumagillin DCH were negative, whereas in the control group an infection of low intensity and 80% prevalence (8 out of 10) was demonstrated. All ten treated eels killed on day 40 of the medication trial were

Table I

Efficacy of Fumagillin DCH against the eel parasite *Myxidium giardi* (laboratory experiments performed in 1987)

Origin of the eels	Beginning of the experiment	End of the experiment	Duration of the experiment (days)	Fish treated with Fumagillin DCH		Control
				0.1%	1%	
Directly from the 1st transport (20 Feb.)	17 March	8 April	22	1 / 17 +	0 / 18	4 / 12 +
Directly from the 1st transport (20 Feb.)	30 March	17 April	19	—	0 / 8	8 / 10 +
Directly from the 1st transport (20 Feb.)	30 March	8 May	40	—	0 / 10	10 / 10 +
Directly from the 2nd transport (21 March)	10 April	8 May	29	—	7 / 20 +	17 / 20 ++
1st transport (20 Feb.) (back from farm)*	17 April	22 May	36	—	8 / 10 +	8 / 10 +++
1st transport (20 Feb.) (back from farm)*	17 April	3 June	48	—	8 / 28 +	10 / 11 +++
2nd transport (21 March) (back from farm)**	25 June	10 August	47	10 / 10 ++	10 / 10 +	10 / 10 +++
2nd transport (21 March) (back from farm)**	25 June	3 September	61	10 / 10 ++	10 / 10 +	10 / 10 +++

+ = low intensity; ++ = medium intensity; +++ = high intensity; numerator: number of infected fish; denominator: number of fish tested ;*: the eels were reared at the farm from 20 February to 15 April; **: the eels were reared at the farm from 21 March to 23 June

negative, while in the control group the prevalence of infection was 100%. In the positive control fish the intensity of infection was low.

In the third experiment eels of the second transport batch were used. These fish had apparently been infected already at the start of the experiment. After feeding 1.0% Fumagillin DCH for 29 days, 7 out of the 20 treated eels were positive, with low intensity of infection. In the untreated controls both intensity and prevalence (17 out of 20) of infection were higher.

The fourth experiment was also evaluated in two parts. In this experiment eels of the first import batch and returned from the eel farm were used. These fish had been infected already at the beginning of the experiment. The prevalence of infection was between 40 and 50% and its intensity was low.

After day 36 of medication, the kidneys of 8 out of 10 eels fed 1.0% Fumagillin DCH were infected by *M. giardi* spores (the intensity of infection was low). In the control group the intensity of infection was very high and the prevalence was the same as in the treated group (8 out of 10). The remaining eels were examined after day 48 of medication. At that time 8 out of the 28 treated eels were infected (with the low intensity already demonstrable at the start of the experiment). At the same time, 10 out of the 11 control eels tested were infected, with an extremely high intensity.

In the fifth experiment, similarly to the fourth one, eels brought back from the eel farm were used. These eels, however, came from the second import batch. At the beginning of the experiment the eels showed 100% prevalence and low intensity of *M. giardi* infection. The experiment was evaluated in two parts.

By day 47 of medication with 1.0% Fumagillin DCH the intensity of infection had not changed as compared to the initial value, and the spore counts demonstrable in the kidneys already at the start of the experiment were present even at the time of evaluation. In fish fed 0.1% Fumagillin DCH, by day 47 of medication the intensity of infection somewhat increased as compared to the initial level. At the same time, in the control fish the intensity of infection increased enormously, i.e. extremely large numbers of spores were demonstrable in the kidneys. At day 61 of medication the same results were obtained as at day 47 for all groups (0.1% Fumagillin DCH, 1.0% Fumagillin DCH, control).

Field trials with eels infected by M. giardi (Table II)

In the first experiment the medicated feed containing 0.1% Fumagillin DCH was fed four times daily over a period of 11 days. The 10 eels examined were negative, whereas 4 out of the 13 control fish had *M. giardi* infection of low intensity. In this experiment the eels had been negative, or latently infected, already at the start.

In the second experiment, eels from the second import batch were used. These eels had been infected, with low prevalence and intensity, already at the beginning of the experiment. After feeding 0.1% Fumagillin DCH four times a day over a period of 9 days, one out of the 10 treated fish was found to have infection of low intensity. At the same time, 3 out of the 10 control fish were infected, with similar intensity as the treated ones. After day 9, the medicated feed containing 0.1% Fumagillin DCH was fed only once, and the normal, non-medicated eel feed an additional three times, daily. After day 21 of medication one out of the 11 treated eels had *M. giardi* infection of low intensity, whereas 5 out of the 20 fish in the two control tanks had low-intensity infection by *M. giardi* spores in the kidney. At day 36 of medication 2 out of the 15

Table II

Efficacy of a medicated diet containing 0.1% Fumagillin DCH against the eel parasite *Myxidium giardi* (field experiments performed in 1987)

Origin of the eels (transport)	Time of the examination	Duration of the experiment (days)	Group treated with Fumagillin DCH	Control group		
				1	2	3
Experiment 1						
1 (20 February)	21 March	11	0/10	4/13+	—	—
Experiment 2						
2 (21 March)	17 April	9*	1/10+	3/10+	—	—
2	29 April	21	1/11	3/10++	2/10++	—
2	14 May	36	2/15+	7/15+++	—	—
2	27 May	48	10/30+	20/20+++	—	—
2	8 June	62	20/20 15+ 5+++	20/20+++	—	—
2	23 June	76	18/20+++	20/20+++	10/10+++	10/10+++
Body mass at the end of experiment 2 ($\bar{X}$)			0.45 g	0.44 g		

+ = low intensity; ++ = medium intensity; +++ = high intensity; numerator: number of infected fish; denominator: number of fish tested; *: the medicated feed was fed four times daily (for 9 days), and subsequently only once daily

treated eels showed infection of low intensity, whereas in the control group 7 out of 15 fish were infected, with spore counts significantly increased since the previous examination. At day 49 of medication 10 out of the 30 treated fish had infection of low intensity. By that time an infection of 100% prevalence and extremely high intensity had developed in the 20 control eels examined. It should be noted that in that period, in late May, the medicated feed was probably not fed regularly: its quantity did not decrease as quickly as it should have. At day 62 of medication the prevalence of infection reached 100% also in the treated group. However, in most eels (15 out of 20) the intensity of infection was markedly lower than in the control. (Prevalence of infection was 100% in the control group as well). On day 76 of the experiment 18 out of the 20 treated eels were positive, and the intensity of infection had increased. The 50 control eels, originating from three control tanks, showed an infection of 100% prevalence and very high intensity.

In the field trial the effect of fumagillin on the eels' body mass gain was also examined. At the end of the experiment the average body mass gain of the treated and control fish was 0.45 and 0.44 g, respectively.

Discussion

Until now, glass eels caught from river mouths at the Atlantic Coast have been thought to be free from infection by the parasite *M. giardi* (Crane and Eversole, 1980). It was assumed that these eels contracted infection only later, at the eel farm, after they had started to feed. This was supported by negative results of the diagnostic examination of eel transports arriving in Hungary. The present experiments have revealed that glass eels are latently infected by certain, hitherto unknown, developmental stages of *M. giardi* which developed into mature stages even under laboratory rearing conditions (where the eels could not contract infection) after some time. It was found that the glass eels had been infected latently (with 100% prevalence) already at the time of importation. There was a difference between the individual eel specimens in the time of spore formation, presumably depending on the time of their infection at the Atlantic Coast. Eels arriving in Hungary on 20 February developed a less intensive infection over a period of 77 days than those arriving a month later, on 21 March, and kept under the same laboratory conditions, in 48 days. A probable explanation for this fact is that, before having been caught, eels of the second transport had spent a longer time in the river mouths of the Atlantic Coast, and were thus longer exposed to the risk of contracting infection from mature eels living there.

Medication trials conducted in aquaria have shown that, when mixed in the feed in 1.0% concentration, Fumagillin DCH successfully prevented the plasmodium and spore formation of *M. giardi* in the latently infected fish. In experiments in which the eels had shown signs of infection already at the start, medication failed to eliminate from the kidney spores that had been present there earlier. Nevertheless, fumagillin did prevent the formation of new spores. This was confirmed by the rise in the prevalence and intensity of infection in the control group. Fumagillin blocks the development of *M. giardi* and prevents the formation of new spores; however, it has no effect on the already existing ones. It should be pointed out that fumagillin prevents the spore formation of the common carp parasite *Sphaerospora renicola*, another myxosporean, as well (Molnár et al., 1987).

Unfortunately, the first field trial, conducted with latently infected eels of the first import batch, was called off after 11 days for technical reasons. The results of this short experiment are promising (the treated group was negative, and the control showed an infection of 30% prevalence and low intensity). However, from these results no far-reaching conclusions can be drawn.

The first three samplings of the second field trial showed that feeding of 0.1% Fumagillin DCH kept *M. giardi* infection at a lower level, in both prevalence and intensity, than in the control groups. In late May, the farm staff's

failure to feed fumagillin regularly resulted in a rise of infection in the treated group: first the prevalence, then also the intensity of infection reached the values obtained for the control groups. These not fully convincing results may be accounted for by the improper feeding of Fumagillin DCH. It is also possible that a single daily dose of Fumagillin DCH in the feed was not sufficient to control the infection. When the normal diet was offered three times a day and the medicated diet once daily, at 11 a.m., the eels presumably failed to consume sufficient amounts of it because of its slightly unpleasant taste. Unfortunately, we could not extend the experiment to more tanks and, thus, were unable to test the effect of other doses.

We have been unable to determine with certainty whether, besides their latent infection brought from the Atlantic Coast, the eels contract infection at the farm and, if yes, how severe this infection is. We do not know whether the substantial increase observed in the intensity of infection can be explained by the gradual development of latent forms, or by the eels' continuous reinfection at the farm.

We can conclude that fumagillin does not have any adverse effect on the body mass gain. However, in toxicity experiments (to be reported later on) we arrived at the conclusion that at a high concentration (5–10%) Fumagillin DCH exerts a negative effect on body mass gain as compared to the control.

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PROTEIN POLYMORPHISM OF SOME AMPHISTOMES AND THEIR HOST TISSUE

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The protein profile of three amphistomes *(Gastrothylax crumenifer, Paramphistomum epiclitum,* and *Gigantocotyle explanatum)* from the buffalo was investigated by polyacrylamide gel electrophoresis and compared with that of the host tissue. Significant differences were observed in the densitometric patterns reflecting the protein polymorphism of parasites and host tissues. *G. crumenifer* and *P. epiclitum* showed 14 and 12 bands respectively (Fig. 1A and 1B), while in the rumen tissue 16 protein fractions were detected (Fig. 1C). *G. explanatum* and liver tissue proteins were separated into 18 and 21 fractions respectively (Fig. 1D and 1E). The number and relative mobilities of protein bands differed considerably not only between *G. crumenifer* and *P. epiclitum* but also between these two amphistomes and the host rumen tissue. Similarly, *G. explanatum* and the liver protein profiles were significantly different from each other. The differences in protein polymorphism are discussed in the light of biochemical taxonomy and host–parasite relationship.

Keywords: Protein polymorphism, amphistome, host tissue, buffalo, biochemical taxonomy, host–parasite relationship

Amphistome taxonomists are facing many difficulties because of the vast number of genera and species described in the past. Till recently, classification has been based on morphological characters. However, owing to the degree of morphological variability in amphistomes, the influence of the worm burden and other host-related factors also contribute to such variations. Sey (1979) synonymized a number of taxa with *Paramphistomum epiclitum* (Fischoeder, 1904) on the basis of histomorphological similarities. Since the protein composition of an organism reflects its genetic constitution, separation of proteins by electrophoresis has proved to be a valuable taxonomic tool. Electrophoretic techniques have been used in separating many *Schistosoma* and *Paragonimus* species (Yoshimura, 1968, 1969).

The present study is an attempt to differentiate various genera of amphistomes parasitizing the water buffalo. The protein polymorphism of the parasites was compared with that of the host tissue in order to reveal the degree of host–parasite relationship.

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Materials and methods

Mature *Gastrothylax crumenifer* and *Paramphistomum epiclitum* were collected from the infected rumen and *Gigantocotyle explanatum* from the liver of water buffaloes *(Bubalus bubalis)* slaughtered at the local abattoir. The parasites and infected host tissues were washed with saline several times in order to remove the debris. Subsequently a 20% and 15% (w/v) homogenate was prepared respectively by using a motor-driven glass teflon homogenizer. After centrifugation the homogenates were processed for polyacrylamide gel electrophoresis according to the method of Davis (1964). Gels were prepared and a 3 mA/gel current was applied. After a complete electrophoretic run the gels were removed from the glass tubes and fixed in perchloric acid, methanol and water (5: 25: 75 v/v) for 30 min and stained for 2 h with Coomassie brilliant blue. The dye (0.04%) was prepared in fixative. After staining, the gels were destained in a solution consisting of glacial acetic acid, methanol and water (5: 20: 75 v/v) until clear protein bands appeared. The gels were stored in 7% acetic acid.

Densitometry of the gels for quantitation of the protein bands was done on a Systronic densitometer (type 201) by placing the gels horizontally on the adaptor. A red filter was used and transmission (per cent) was recorded at 1 mm intervals along the length of the gel up to the bromophenol blue marker. The densitometer had been calibrated by using an unstained portion of the gel.

Results and discussion

The results obtained on the protein polymorphism of the parasite and host tissue with respect to electric mobility are summarized in Table I.

Table I

Protein polymorphism of some amphistomes and host tissue

Parasite/Host tissue	n	Number of bands			Total
		anodal region	mid-gel region	cathodal region	
Gastrothylax crumenifer	3	8	1	5	14
Paramphistomum epiclitum	3	6	2	4	12
Rumen tissue	3	9	2	5	16
Gigantocotyle explanatum	3	7	2	9	18
Liver tissue	3	10	2	9	21

n = number of replications

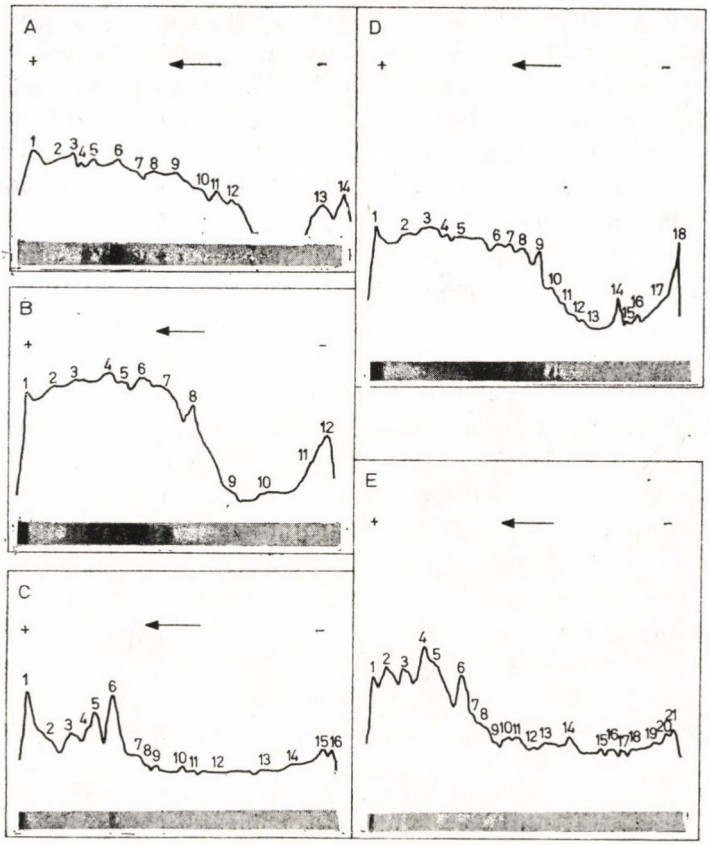


Fig. 1. Electropherograms with corresponding densitometric scans of protein profile. Arrow indicates the direction of protein migration. Numbers on the scan represent the bands. A: *Gastrothylax crumenifer;* B: *Paramphistomum epiclitum;* C: buffalo rumen tissue; D: *Gigantocotyle explanatum;* E: buffalo liver tissue

Significant differences were observed in the densitometric patterns reflecting the protein polymorphism of parasites and host tissues. *G. crumenifer* and *P. epiclitum* showed 14 and 12 bands respectively (Fig. 1A and 1B), while in the rumen tissue 16 protein fractions were detected (Fig. 1C). *G. explanatum* and liver tissue proteins were separated into 18 and 21 fractions respectively (Fig. 1D and 1E). However, the number of anodal and cathodal bands and their relative mobilities were also different in trematodes and in respective host tissues (Table I).

The number and relative mobilities of protein bands differed considerably not only between *G. crumenifer* and *P. epiclitum* but also between these two amphistomes and the host rumen tissue. Similarly, *G. explanatum* and the liver protein profiles were significantly different from each other. Thus it could be inferred that each amphistome as well as the host tissue has a specific num-

ber and a particular migration pattern of protein bands. Many workers have also separated and synonymized several controversial species belonging to different genera of trematodes on the basis of similarities and dissimilarities in their protein spectrum (Yoshimura et al., 1970; Klimenko and Velichko, 1972). Osikovski et al. (1978) investigated the electrophoretic patterns of the water-soluble proteins of *P. microbothrium*, *P. cervi* and *P. microbothrioides*. *P. cervi* showed 16, *P. microbothrium* 18, and *P. microbothrioides* 19 bands. The individual fractions had different electrophoretic mobility characteristic of the species tested. Similarly, the protein profile of the three *Paramphistomum* species has many aspects in common. The present study is in agreement with earlier findings as far as the specificity in the protein profile of a particular species is concerned. Such a study could prove to be a useful tool in the field of biochemical taxonomy of a variety of trematodes.

The similarities and differences noted in the present study in the protein profiles among the three amphistomes and their respective host tissues would also provide some basic knowledge regarding the host–parasite relationship. Furthermore, certain protein bands of the parasites showing dissimilarity with those of the host tissue can be used as targets for chemotherapeutical control. Such variations in the protein profile may further be exploited for pharmacological and immunological studies.

However, further studies are required to investigate differences in protein polymorphism which is a consequence of differences in gene expression.

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DISPOSITION KINETICS AND DOSAGE REGIMEN OF 2-FORMYL 1-METHYL PYRIDINIUM OXIME (2-PAM) IN HEIFERS

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Disposition kinetics and dosage regimen of 2-PAM were investigated in heifers after its intravenous administration (30 mg/kg body mass). The peak plasma level of 2-PAM (200.9 $\pm$ 12.8 $\mu g \cdot ml^{-1}$) was reached at 1 min and therapeutic concentration was maintained up to the 6th hour. The distribution half-life and elimination half-life were calculated to be 0.091 $\pm$ 0.008 h and 2.49 $\pm$ 0.13 h, respectively. The values of $V_{d(area)}$ and total body clearance (Cl_B) were 1.438 $\pm$ 0.072 $L \cdot kg^{-1}$ and 382.7 $\pm$ 58.7 $ml \cdot kg^{-1} \cdot h^{-1}$, respectively. 2-PAM bound to the plasma proteins of heifers to the extent of 48.4 $\pm$ 1.97 per cent. The binding capacity of 2-PAM to plasma proteins and the dissociation rate constant of the protein–drug complex were 3.85×10^{-6} mol $\cdot g^{-1}$ and 2.57×10^{-4} mol, respectively. To maintain the therapeutic plasma concentration in heifers, 2-PAM administration is to be repeated at a dose rate of 26 mg/kg body mass 6 h after the first treatment.

Keywords: 2-PAM, disposition kinetics, dosage regimen, *in vitro* plasma protein binding, heifer

Antidotal treatment of organophosphate poisoning involves atropine and acetylcholinesterase (AChE) reactivators. 2-formyl 1-methyl pyridinium oxime (2-PAM) is used widely against organophosphate intoxication in man and animals (Warriner et al., 1977; Taylor, 1980; Hatch, 1982). The efficacy of treatment with AChE reactivators depends on the repeated administration of these drugs in optimum dose and at suitable time intervals, determined from their disposition kinetics studies. Although the disposition kinetics of different AChE reactivators has been studied in man and animals (Sidell and Groff, 1971; Srivastava et al., 1985; Srivastava and Malik, 1986), such investigations on 2-PAM are lacking.

Accordingly, the objective of the present study was to investigate the disposition kinetics and dosage regimen of 2-PAM in heifers. *In vitro* plasma protein binding of 2-PAM was also studied.

Materials and methods

Disposition kinetics

Experiments were performed on healthy heifers with an average body mass of 180–216 kg. The animals were purchased from the local market and housed in a departmental animal shed prior to the start of the experiments.

After withdrawing the control blood sample, 2-PAM (Aldrich Chemical Co., Milwaukee, Wisconsin, USA) was administered as 8 per cent solution in isotonic saline into the left jugular vein of the animals at a dose level of 30 mg/kg body mass. (The dose of reactivator refers to base). Blood samples were collected from the right jugular vein into heparinized glass centrifuge tubes at 1, 2.5, 5, 7.5, 10, 15 and 30 min and 1, 1.5, 2, 3, 4, 5, 6 and 8 h after drug administration. Erythrocytes and plasma were separated at room temperature. The concentration of 2-PAM in the blood plasma and erythrocytes was measured by an UV spectrophotometric method as described by Creasy and Green (1959). The plasma concentration–time profile of 2-formyl 1-methyl pyridinium oxime was used to calculate various pharmacokinetic parameters (Gibaldi and Perrier, 1975).

In vitro plasma protein binding

In vitro binding of 2-PAM to plasma proteins of heifers was determined by employing an equilibrium dialysis technique (Kunin et al., 1959). A dialysis bag of 4 Å pore size was used in the present investigation. After estimating free and bound drug the binding capacity of plasma proteins to 2-PAM and the dissociation rate constant of the protein–drug complex were determined by the procedure of Pilloud (1973).

Results and discussion

A semilogarithmic plot of decline in the plasma concentration of 2-PAM is shown in Fig. 1. The data were best fitted to a two-compartment open model and adequately described by the equation $CP = Ae^{-\alpha t} + Be^{-\beta t}$. At 1 min of injection the peak plasma concentration of 2-PAM was $200.9 \pm 12.8\ \mu g \cdot ml^{-1}$ which declined to $1.48 \pm 0.45\ \mu g \cdot ml^{-1}$ at 8 h of administration. The therapeutic plasma level of 2-PAM has been reported to be $\geq 4\ \mu g \cdot ml^{-1}$ (Sundwall, 1961). In this study, this concentration of 2-PAM was maintained from 1–240 min of its administration. Intravenous administration of AChE reactivator is the most reliable means of treating organophosphate-poisoned animals, as an initially high drug level in the central compartment is required to obtain a favourable diffusion gradient.

The therapeutic efficacy of 2-PAM depends on its penetration into erythrocytes. At different blood concentrations, 2-PAM penetrated into erythrocytes to the extent of 3.84–16.4 per cent of the total blood concentration (Table I). No correlation between the concentration of drug in the blood and the extent of its penetration into erythrocytes could be established. The good penetration of 2-PAM into erythrocytes reflects that 2-PAM may be

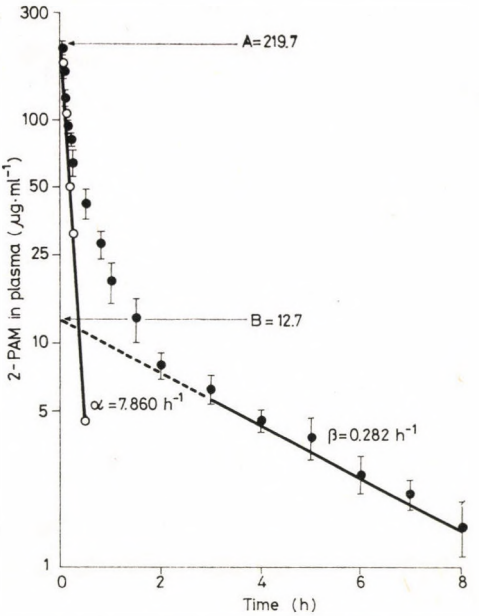


Fig. 1. Semilogarithmic plot of plasma concentration–time profile of 2-PAM in heifers following a single intravenous dose (30 mg/kg). Values given are mean ± SE of the results obtained for 5 animals. Distribution (α) and elimination (β) phases are represented by least square regression lines. The calculated points (0) of the distribution phase were obtained by the feathering technique

beneficial in the treatment of organophosphate insecticide poisoning in heifers. Organophosphate insecticides are known to inhibit the erythrocyte cholinesterase to a greater extent than other esterases (Srivastava et al., 1984).

Various disposition kinetic parameters of 2-PAM are summarized in Table II. The high value of rate constant α (7.86 ± 0.74 h^{-1}) indicated that 2-PAM is rapidly distributed in peripheral compartment of heifers. The rapid distribution of 2-PAM is further substantiated by the high ratio of K_{12}/K_{21}

Table I

Levels of 2-PAM in blood and erythrocytes of heifers following a single intravenous dose of 30 mg/kg body mass

Time after 2-PAM administration (h)	Blood ($\mu g \cdot ml^{-1}$)	Erythrocytes (% of blood concentration)
0–1	14.5—150.3	11.6 ± 1.32
1–2	5.54—7.32	3.84 ± 0.11
2–4	2.52—4.62	14.4 ± 0.84
4–8	0.53—2.52	15.4 ± 2.12

Values given are mean ± SE of the results obtained for 5 animals

Table II

Pharmacokinetics of 2-PAM in heifers following a single intravenous dose of 30 mg/kg body mass

Kinetic parameter	Unit	Mean ± SE	Range
C_P^0	$\mu g \cdot ml^{-1}$	223.4 ± 7.94	217.5–250.9
A	$\mu g \cdot ml^{-1}$	219.7 ± 8.72	201.7–240.6
α	h^{-1}	7.860 ± 0.740	6.444–9.623
$t_{1/2\alpha}$	h	9.091 ± 0.008	0.072–0.108
B	$\mu g \cdot ml^{-1}$	12.7 ± 1.38	10.3–16.5
β	h^{-1}	0.282 ± 0.015	0.249–0.318
$t_{1/2\beta}$	h	2.49 ± 0.13	2.18–2.78
K_{12}	h^{-1}	4.278 ± 0.642	2.725–5.369
K_{21}	h^{-1}	0.713 ± 0.079	0.547–0.893
K_{12}/K_{21}	Ratio	5.919 ± 0.347	4.986–6.666
K_{el}	h^{-1}	3.151 ± 0.308	2.531–3.854
$t_{1/2Kel}$	h	0.226 ± 0.022	0.179–0.252
AUC	$\mu g \cdot ml^{-1} \times h$	75.74 ± 7.01	57.32–87.34
V_c	$L \cdot kg^{-1}$	0.129 ± 0.004	0.119–0.138
$V_{d(area)}$	$L \cdot kg^{-1}$	1.438 ± 0.072	1.318–1.646
$V_{d(B)}$	$L \cdot kg^{-1}$	2.231 ± 0.390	1.32–2.913
$V_{d(ss)}$	$L \cdot kg^{-1}$	0.899 ± 0.071	0.0876–1.042
Cl_B	$ml \cdot kg^{-1} \cdot h^{-1}$	382.7 ± 58.7	243.5–523.4
td	h	7.21 ± 0.39	6.33–8.08
T/P	Ratio	10.1 ± 0.57	8.55–11.12

[a] Kinetic parameters are described by Gibaldi and Perrier (1975).
[b] Values given are mean ± SE of the results obtained for 5 animals

(5.919 ± 0.347). Clinically it is of utmost importance to determine the extent of drug distribution rather than the rate of distribution. The calculated apparent volume of distribution (1.44 ± 0.07 $L \cdot kg^{-1}$) indicates that there is good distribution of 2-PAM in body fluids and tissues. The rather high T/P ratio (10.1 ± 0.57) further confirmed the extent of penetration of 2-PAM into body fluids and tissues of heifers. The identical values of $V_{d(area)}$, $V_{d(B)}$ and $V_{d(ss)}$ are consistent with the findings of Notori (1973) and suggest that the methods of calculation have no influence on the volume of distribution of the drug.

The elimination half-life of 2-PAM in heifers (2.49 ± 0.13 h) established in this study is similar to the $t_{1/2\beta}$ of 2-PAM in male rats. The half-life of 2-PAM was reported to be 88 min in female rats and 124 min in male rats (Das Gupta et al., 1979). As compared to heifers a shorter elimination half-life of 2-PAM (56.76 min) was reported in man (Jager et al., 1958; Sidell and Groff, 1971; Swartz and Sidell, 1974).

Table III shows the capacity of plasma proteins to bind 2-PAM (β_i) and the dissociation rate constant of the protein–drug complex (K_β). At different concentrations of 2-PAM the values of β_i and K_β were calculated to be $3.85 \times 10^{-6} \pm 0.137 \times 10^{-6}$ and $2.57 \times 10^{-4} \pm 0.186 \times 10^{-4}$ mol respectively. Overall, 48.4 $\pm$ 1.97% of 2-PAM was bound to the plasma proteins of heifers. Plasma protein binding of a drug is an important index to assess the active fraction of drug in the blood and tissue compartment. It has been concluded that low to moderate (<85%) degrees of protein binding have little or no influence on the kinetic disposition of drug (Baggot, 1982). On the basis of β_i and K_β, it can be established that *(i)* the binding of 2-PAM to plasma proteins of heifers is weak because of the negligible value of β_i and *(ii)* the protein binding of 2-PAM is reversible because of the higher value of the dissociation rate constant of the protein–drug complex than the binding capacity of plasma protein.

The ultimate objective of a disposition kinetic study is to determine the dosage regimen. With a convenient and suitable dosage interval, calculation of the maintenance dose ($\dot{D}$) is based on the minimum effective concentration (MEC) of 2-PAM and is calculated by the formula.

$$\dot{D} = MEC \cdot V_{d(area)} (e^{+\beta t} - 1)$$

Taking 6 h as convenient and suitable dosage interval, with a minimum therapeutic plasma level of 4 $\mu g \cdot ml^{-1}$ and usiug the values of β and $V_{d(area)}$ of Table I, the optimal dose was determined. Based on these parameters, it is suggested that in heifers 2-PAM treatment is to be repeated at the dose rate of

Table III

In vitro binding of 2–PAM to pasma proteins of heifers

Concentration of 2-PAM ($\mu g \cdot ml^{-1}$)	Parameter[a]		
	B_i (mole·g^{-1})	K_B (mole)	Extent of binding (%)
2	4.15×10^{-6}	2.95×10^{-4}	48.3
5	3.81×10^{-6}	1.85×10^{-4}	46.68
10	$4.15 \times 10^{-}{}_{9}$	2.66×10^{-4}	45.2
25	3.13×10^{-6}	2.95×10^{-4}	52.6
50	3.95×10^{-6}	3.18×10^{-4}	54.5
100	4.08×10^{-6}	2.25×10^{-4}	47.8
200	3.68×10^{-6}	2.16×10^{-4}	43.5
Mean	3.85×10^{-6}	2.57×10^{-4}	48.36
$\pm$ SE	0.137×10^{-6}	0.186×10^{-4}	1.97

For each concentration, values given are the mean of four experiments.
[a] Various parameters were calculated by the method of Pilloud (1973)

25.5 (26) mg/kg body mass 6 h after the first treatment. The loading dosage level of 2-PAM employed in the present investigation is quite comparable to the dosage given for the treatment of organophosphate poisoning in man and animals (Jager et al., 1958; Wright et al., 1966; Taylor, 1980).

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PROTEIN-INDUCED CHANGES IN THE RESPONSE OF PLASMA CORTISOL OF LAMBS TO HEAT STRESS

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The present study was conducted to show whether or not protein intake can induce changes in the plasma cortisol concentration of lambs exposed to acute and chronic heat stress.

Four groups, each comprised of four Merino lambs, were used. Two groups were kept at 18 °C and the others were subjected to an ambient temperature of 32 °C. The animals received a diet supplying each of them with 98.8 and 154.4 g crude protein daily in the first and second group, respectively, at each environmental temperature, and with 6.9 MJ metabolizable energy daily in each group.

Cortisol concentration was determined by radioimmunoassay to study the effect of protein intake and acute (3 days at 32 °C) and chronic (30 days at 32 °C) heat stress on cortisol concentration.

On the third day of heat exposure cortisol concentration was nearly 2.5 times that measured at comfort temperature, at both protein intake levels. When exposed to 32 °C for one month, the lambs had a significantly decreased plasma cortisol concentration at both levels of protein intake. The study showed a positive effect of increasing protein intake and heat stress on the concentration of plasma cortisol in sheep.

Keywords: Protein intake, plasma cortisol, heat stress, lamb

The adrenal gland is known to be affected by the protein content of the diet. A review of the literature of adrenal hypertrophy revealed that many of the circumstances in which such hypertrophy occurs are characterized by either a relative or an absolute increase in the rate of protein catabolism. Also, there is evidence that administration of certain amino acids can influence the functional activity of the adrenal cortex. It was suggested that the pituitary–adrenal system was more active in animals under stress if they were fed a high-protein diet, and this effect was attributed to an enhanced secretion of adrenocorticotropin.

Although the effect of heat stress on cortisol concentration has been evaluated, the probable action that protein may exert in modifying this effect in ruminants has not been clarified yet. Therefore, the present study was conducted to show the changes induced in cortisol concentration by different levels of protein intake when the lambs were exposed to acute or chronic heat stress.

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Materials and methods

Sixteen 3-month-old Merino lambs, with an initial body mass of 26 ± ± 1.5 kg each, were assigned randomly to four groups and housed in individual pens. Two groups were kept at 18 ± 2 °C and the other two were subjected to an ambient temperature of 32 ± 1 °C.

The animals were fed 750 g pelleted diet once daily at 7:00 a.m. and an additional 200 g of hay at noon. The diet provided each animal with 98.8 g (medium protein intake) or 154.4 g (high protein intake) crude protein daily in the first and second group, respectively, at each environmental temperature. The protein content of the diet was adjusted on the basis of chemical analysis of each ingredient used. The metabolizable energy content of the diet was calculated from N. R. C. tables (1968) to supply each animal with 6.9 MJ daily in each group. According to Rattray et al. (1973), this calculated energy level was expected to meet the net energy requirements for maintenance and to promote a daily gain of about 100 g.

Table I shows the ingredient composition of the experimental diets. Alfalfa meal was used in the same proportion in both diets and soybean meal replaced corn in order to change protein without altering the energy content of the diets.

Heat exposure took place after the animals had been kept at an ambient temperature of 18 °C and fed a medium or high-protein diet for one week. Blood was collected by venipuncture every second day of that week in order to make the animals familiar with the staff and the sampling technique. Plasma cortisol concentration of blood collected at the third sampling (one day before heat exposure) was used as control for that measured in blood taken at the fifth sampling (on the third day of heat exposure). By this method the response of cortisol concentration to acute heat stress was monitored. After

Table I

Composition of the rations fed to lambs (in %)

Ingredient	Medium protein diet	High protein diet
Alfalfa meal	20	20
Corn	66	46
Soybean	10	30
Mineral and vitamin mixture	4	4
Total:	100	100
Crude protein (%)	13.18	20.58
Metabolizable energy (MJ/kg)	9.186	9.285
Daily crude protein intake (g)	98.85	154.35
Daily metabolizable energy intake (MJ)	6.89	6.96

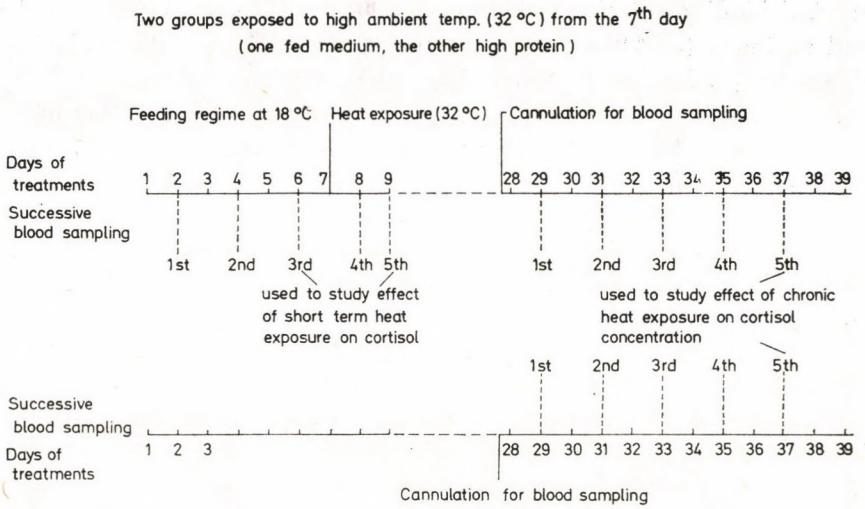


Fig. 1. Design of the experiment

three weeks of heat exposure, catheters were inserted into the jugular vein for blood sampling which was carried out every second day. One week was considered sufficient for the animals to recover from the surgical load and to get accustomed to the sampling procedure. Plasma cortisol concentration was measured in blood from lambs kept at 18 ° C and in that from lambs kept at 32 °C for one month to study the effect of long-term heat exposure on cortisol concentration. The design of the experiment is shown in Fig. 1.

Assay of plasma cortisol concentration

Cortisol concentration was determined by radioimmunoassay (RIA) as follows.

Extraction. 3000 cpm ^{3}H-cortisol was dried in a conical stopper glass tube of 20 ml volume, which was used to assess recovery. A 300 μl volume of plasma was shaken with 3 ml dichloromethan for 2 min, centrifuged at 3000 rpm for 10 min, and the dichloromethan phase was transferred into a clean glass tube and evaporated to dryness in waterbath (37 °C) under a stream of air.

RIA. The dried extract was dissolved in 300 μl absolute ethanol using a vortex mixer and the solution was divided into three aliquots. The first 100 μl aliquot was transferred into a cuvette and 5 ml scintillation solution (3 parts Bray solution and 1 part Triton X-100) was added to it to measure the recovery. The second 100 μl volume was transferred into a polyethylene tube for RIA. The remaining 100 μl volume was evaporated and dissolved in 200 μl

^{3}H-cortisol binding solution (6000 cpm), 200 μl phosphate buffer and 1 : 800 diluted antibody (at 4 °C) using a vortex mixer for 30 sec. The solution was incubated at + 37 °C for 30 min and at + 4 °overnight.

After incubation, separation was carried out by adding 500 μl of a mixture of Norit A + Dextran T_{70} (2 g Norit A and 200 mg dextran T_{70} dissolved in 1 litre of phosphate buffer), allowed to stand for 10 min at 4 °C, then centrifuged at 300 rpm for 10 min at 4 °C.

A 500 μl volume of the supernatant was added to 5 ml of the scintillation solution in a cuvette and radioactivity was measured for 10 min with a Packard Tricarb 3375 scintillation spectrophotometer.

Counting. The values of bound and total radioactivity were calculated, in per cents, from a standard curve. The calculated values were corrected by the measured recovery coefficient to obtain the actual amount of cortisol in 1 ml of plasma.

Results

Response of cortisol concentration to heat stress

On the third day of heat exposure the lambs had significantly increased levels ($P < 0.01$) of cortisol. At high temperature cortisol concentration was nearly 2.5 times higher than that measured at comfort temperature at both protein intake levels (Table II).

Lambs exposed to moderate heat stress (32 °C) chronically, for one month, had a significantly ($P < 0.01$) decreased plasma cortisol concentration at both medium and high protein intake (Table III).

Table II

Influence of protein intake on cortisol concentration (ng/ml) when lambs were exposed to 18 °C for 3 days

Cortisol concentration, ng/ml	Temperature			
	18 °C		32 °C	
	Medium protein intake	High protein intake	Medium protein intake	High protein intake
	3.06	3.30	7.08	8.41
	3.44	3.42	7.95	9.23
	2.83	3.86	6.00	10.12
	3.13	4.21	7.54	11.00
$\bar{X}$	3.12^{A}	3.69^{B}	7.14^{AB}	9.69^{AB}
$\pm$ SD	0.25	0.42	0.84	1.12

Values having a common letter in their superscript are significantly different

Table III

Plasma cortisol concentration (ng/ml) of lambs fed two different protein levels and kept at 18 °C or 32 °C for one month

Cortisol concentration, ng/ml	Temperature			
	18 °C		32 °C	
	Medium protein intake	High protein intake	Medium protein intake	High protein intake
	2.89	3.95	1.90	2.40
	2.71	3.40	2.45	2.43
	3.04	3.04	1.43	2.66
	2.55	3.27	2.30	2.77
$\bar{X}$	2.80^{Aa}	3.42^{Ba}	2.02^{Ab}	2.57^{Bb}
$\pm$ SD	0.21	0.39	0.46	0.18

Values having a common letter in their superscript are significantly different (A, B = $= P < 0.01$; a, b = $P < 0.05$)

Effects of protein intake on cortisol concentration

High protein intake had a definite effect on the plasma cortisol concentration of growing lambs at both short-term and chronic heat stress. It significantly ($P < 0.01$) increased the plasma cortisol concentration of lambs exposed to 32 °C for 3 days (Table II) and caused a significant rise ($P < 0.05$) in the cortisol concentration of animals exposed to chronic heat stress or kept at 18 °C (Table III).

Discussion

Effect of blood sampling procedure on cortisol concentration

Cortisol values for sheep seem to vary considerably in the different laboratories even when similar techniques are used for the measurements.

Bassett and Hinks (1969) found that increases in plasma corticoids were caused by successive venipuncture and the increase was less expressed in animals conditioned to handling as compared to untrained animals. Similarly, Reid and Mills (1962) observed that plasma cortisol response was less pronounced when trained animals were transported as compared to grazing animals. These observations, along with the data reported by Willett and Erb (1972), suggest that the most practical approach is to accustom the animals to frequent handling and to the experimental procedure prior to experimentation. This approach was used in our experiments.

Data in Table IV, collected from Tables I and II, show that the differences between the two base values serving as control for short-term and long-

Table IV

Effect of the blood sampling procedure on cortisol concentration of lambs kept at 18 °C and fed two different protein levels

Cortisol concentration, ng/ml	One week on feed regime and blood sampling by venipuncture		One month on feed regime and sampling through cannula	
	Medium protein intake	High protein intake	Medium protein intake	High protein intake
	3.06	3.30	2.89	3.95
	3.44	3.42	2.71	3.40
	2.83	3.86	3.04	3.04
	3.13	4.21	2.55	3.27
$\bar{X}$	3.12^a	3.69^a	2.80^b	3.42^b
$\pm$ SD	0.25	0.42	0.21	0.39

Values having a common letter in their superscript are significantly different ($P < 0.05$)

term heat exposure were not significant ($P > 0.05$). This shows that the animals were well accustomed to the experimental procedure.

The plasma cortisol value (2.8 ng/ml) obtained by us in growing sheep accustomed to the technique, kept at neutral temperature (18 °C) and fed the medium protein level, is similar to the values (2–4 ng/ml) measured by Tilton et al. (1975) in mature ewes, and slightly higher than those reported by others (between 1.8 and 2.0 ng/ml; Saba, 1964; Paterson, 1964; Paterson and Harrison 1967; and Paterson and Hills, 1967). The value obtained by us is, however, much higher than 0.5–1.0 ng/ml, a value reported by some authors in sheep, under different conditions (Bassett, 1963; Lindner, 1964; Coghlan and Scoggins, 1967; Bassett and Hinks, 1969).

The wide variations in plasma cortisol concentration of sheep reported in the literature comprise the effect of many variables including the techniques used, feeding, environmental factors, degree of stress, or adaptation of the animals to the sampling technique.

Effect of protein intake on plasma cortisol concentration

The influence of excess protein intake on the pituitary–adrenal axis has not been widely studied in farm animals.

The positive effect of high protein intake on plasma cortisol level of sheep in this study (Tables II and III) obeys the general trend of protein effect reported by Kovács and Korpássy (1952) who suggested that in rats under stress the pituitary–adrenal system was more active if the animals were given high-protein diets. This may explain the finding that the rise of the

cortisol level in animals exposed to acute heat stress was more expressed when the animals consumed more protein (Table II). A higher secretion rate of ACTH caused by higher protein levels in the rat (Munro et al., 1962; Munro, 1964; Munro et al., 1965) might have been the case in lambs of this study, consuming 150 g instead of 100 g crude protein daily. This increased protein intake might have led to enhanced activation of the adrenals to secrete more cortisol at both temperatures (Tables II and III).

The exact mechanism via which protein or amino acids exert their effect on the pituitary gland to release ACTH has not been elucidated yet. However, Tegler and Schuelke (1981) showed that increased protein resulted in higher glucocorticoid-binding capacity, leading to a decrease in free cortisol. Since only free cortisol can exert a feedback effect on the pituitary, high protein intake may indirectly affect ACTH release by decreasing the feedback effect of cortisol.

The indirect effect of dietary protein on cortisol concentration might be mediated through its influence on insulin since insulin hypoglycaemia activates the whole hypothalamus–pituitary–adrenal axis (Bethge et al., 1967) and plasma insulin of ruminants was shown to be related to protein intake (Bassett et al., 1971; Borger et al., 1973; Martin et al., 1979).

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ISOLATION AND CHARACTERIZATION OF RETICULOENDOTHELIOSIS VIRUS FROM TURKEYS

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Reticuloendotheliosis virus (REV) infection was demonstrated in laying flocks of an integrated turkey breeding organization which exhibited low levels of mortality from leukotic tumours, sporadic fowl pox like lesions and fluctuating egg fertility. Natural mortality from lymphoid neoplasms in the liver, spleen, pancreas and small intestine occurred after 20 weeks of age. Histologically the tumours were composed of a moderately pleomorphic population of lymphoblastoid cells. There was no bursal involvement, and the peripheral nerves lacked mononuclear endoneural infiltrations. Skin lesions were of fowl pox origin and not a cutaneous manifestation of reticuloendotheliosis. Antibodies to REV were demonstrated by the indirect immunofluorescence test in 2 to 50% of random serum samples from five of the seven examined laying flocks. Egg infertility problems observed in some flocks could not be directly related to REV infection.

In chick embryo fibroblast cultures, chloroform sensitive viruses of about 100 nm in diameter with typical C-type morphology and RNA-directed DNA polymerase activity were isolated from plasma samples and lymphoid tumours of turkeys over 28 weeks of age. The virus isolates, designated as strains REV-H-10 and REV-H-16, were culturally and antigenically similar to the prototype REV strain T, and were free of contamination with exogenous avian leukosis-sarcoma viruses by cell-culture assay.

In embryonally, neonatally or contact infected turkeys, cell-free virus isolates produced a chronic neoplastic disease similar in gross and microscopic appearance to that seen in the natural disease, with latent periods of 91 to 237 days. Tumour mortality of the 1-day-old inoculated poults was 16% during a 34-week experimental period, and 20% of the tumour-bearing turkeys had bursal involvement. Turkeys infected by contact exposure from 1 day of age developed lymphomas at a lower (5.6%) frequency, whereas those infected at 4 weeks of age were refractory to tumour development. REV could be reisolated from 57% of birds that died with tumours. REV viraemia and antibodies to REV were detected in a number of turkeys from all experimentally infected groups. Neonatally infected turkeys were deficient in the secondary antibody response to sheep red blood cells and *Brucella abortus* and in mitogen-induced blastogenesis of blood lymphocytes at 30 to 34 weeks of age.

Keywords: Turkey, avian reticuloendotheliosis virus, isolation and characterization, natural and experimental infection, pathology, immune response

The most common naturally occurring neoplastic disease of turkey *(Meleagris gallopavo)*, leukosis, is associated with infection of either reticuloendotheliosis virus (REV) belonging to the Retroviridae family or an unclassified retrovirus called lymphoproliferative disease virus (Biggs et al., 1978; Biggs, 1980). The prototype virus of the REV group of avian C-type retroviruses (Purchase et al., 1973) is strain T which was isolated from a turkey with lymphoproliferative lesions (Robinson and Twiehaus, 1974). This virus strain

induces an acute reticulum cell neoplasia in a number of avian species leading to death within one to two weeks after infection (Taylor and Olson, 1972; Theilen et al., 1966). It has been shown (Hoelzer et al., 1979) to consist of two viruses: a replication-defective virus with v-rel oncogene (Wong and Lai, 1981) which transforms avian cells in culture and is responsible for the acute oncogenicity, and a nondefective helper virus which causes either nonneoplastic disorders, including runting, bursa and thymus atrophy, general immunosuppression, peripheral nerve enlargement, abnormal feather development, enteritis, proventriculitis, anaemia, liver and spleen necrosis (Jackson et al., 1977; Koyama et al., 1976; Ludford et al., 1972; McDougall et al., 1978; Mussman and Twiehaus, 1971; Trager, 1959; Witter et al., 1970), or a chronic neoplastic disease with or without the involvement of the bursa of Fabricius (Witter and Crittenden, 1979; Witter et al., 1981, 1986).

Since the original isolation of the T strain of REV, additional nondefective REV strains have been isolated from turkey flocks with a history of mortality from neoplastic disease (McDougall et al., 1978; Paul et al., 1976; Sarma et al., 1975; Solomon et al., 1976; Witter and Glass, 1984). The present paper reports the isolation of a C-type retrovirus identified as a new member of the REV group from adult breeding turkey flocks experiencing low levels of neoplastic mortality, sporadic fowl pox and egg infertility with great economic impact. Some characteristics and pathogenicity of the new virus isolates are also reported.

Materials and methods

Experimental turkeys

Turkey hatching eggs and 1-day-old poults were obtained from a parent breeding turkey flock known to be free of REV. The poults were reared on the floor in animal rooms and were fed on commercial turkey grower and breeder rations. Feed and water was made available ad libitum to all birds.

Purification of turkey IgG

Turkey immunoglobulin G (IgG) was purified from egg yolk. Egg yolk globulin, separated as described (Jensenius et al., 1981), was dialysed against Tris (hydroxymethyl) aminomethane – HCl + 0.03 M NaCl, pH 7.5, buffer and applied to a DEAE-Sephacel (Pharmacia Biotechnology International AB, Uppsala, Sweden) column equilibrated with the same buffer. The IgG fraction that passed straight through the column was further fractionated by preparative agarose gel electrophoresis (Johansson, 1972) to obtain pure IgG.

Production of fluorescein-labelled rabbit antiserum to turkey IgG

Rabbits were injected intradermally with turkey IgG in a dose of 50–100 μg/kg body mass emulsified in incomplete Freund's adjuvant (Difco Laboratories, Detroit, U.S.A.) six to eight times fortnightly, and bled out 10 to 14 days after the last inoculation. The IgG fraction of the obtained immune serum was isolated (Harboe and Ingild, 1973) and labelled with fluorescein-isothiocyanate (FITC, Isomer 1, Serva Feinbiochemica GmbH and Co., Heidelberg, Germany) at a rate of 16 μg dye/mg IgG according to Goldman (1968). The conjugate was characterized as described by Holborow and Johnson (1967). It contained 20 mg/ml of protein, had a FITC/protein molar ratio of 3.8 and had a standard gel diffusion precipitation titre of > 64 against turkey IgG. It was used at 1 : 40 dilution for indirect immunofluorescence (IF) test.

Immune sera

Chicken anti-REV-T immune serum was obtained from Intervet, Ltd (Netherlands), rabbit anti-avian myeloblastosis virus (AMV) immune serum from Dr. E. J. Smith (Regional Poultry Research Laboratory, East Lansing, U.S.A.) and FITC-conjugated swine anti-rabbit globulin from SEVAC (Prague, Czechoslovakia). Production of FITC-labelled rabbit antiserum to chicken immunoglobulins has been described previously (Drén et al., 1988).

Cell cultures

Cultures of chick embryo fibroblasts (CEF) were prepared and maintained by the method described by Temin and Rubin (1958), from 10-day-old embryos which were derived from the specific-pathogen-free White Leghorn flock of Phylaxia Veterinary Biologicals Co. (Budapest, Hungary).

Viruses

The REV-H-1 strain was isolated from lymphoreticular tumours of pheasants (Drén et al., 1983). The nondefective REV-T strain was obtained from Dr. R. L. Witter (Regional Poultry Research Laboratory, East Lansing, U.S.A.) and the HPRS-F42 strain of avian lymphoid leukosis virus from Dr. L. N. Payne (Houghton Poultry Research Station, Houghton, England). All virus strains were propagated in CEF cultures and used as cell-free fluids from the 3rd to 6th tissue culture passage.

Virus isolation

For virus isolation cell suspensions were prepared aseptically from suspected tumourous organs and/or the spleens in Ham's F-10 medium (Flow

Laboratories Ltd, Irvine, Scotland) containing 10% (v/v) newborn calf serum, 10% (w/v) tryptose phosphate broth (Difco Laboratories, Detroit, U.S.A.) and 7.5% (v/v) dimethyl sulphoxide, and stored in the gaseous phase over liquid nitrogen for further processing. Cell viability was determined by the trypan-blue exclusion test. Heparinized (50 units/ml) whole blood was taken aseptically from turkeys by venipuncture. Plasma was separated from the blood cells by centrifugation and stored at −70 °C until tested.

Duplicate 18- to 24-hour monolayers of CEF in 60 mm plastic Petri dishes (Falcon Plastics, Los Angeles, U.S.A.) were either inoculated into the medium with about 10^6 cells/0.2 ml or the medium was removed and 0.2 ml plasma was inoculated onto the drained monolayers. In the latter case, after one hour of adsorption at 37 °C, the cells were refeeded with growth medium supplemented with 5 units/ml heparin. Control cultures were either inoculated with the same dose of spleen cells from normal healthy turkeys or remained uninoculated. The cultures were maintained under liquid medium at 37 °C in a 5% (v/v) CO_2 humidified atmosphere for 17 days, with two intermediate passages, and examined for REV and avian leukosis-sarcoma virus (ALSV) group-specific antigens by the indirect IF technique as described previously (Drén et al., 1988) using chicken antiserum to REV-T strain and rabbit antiserum to AMV and the appropriate FITC-labelled anti-immunoglobulin reagents, respectively.

Test for sensitivity to chloroform

Sensitivity of the new virus isolates to 5% (v/v) chloroform was tested according to Lukert (1980). Residual infectivity of the chloroform-treated virus isolates was tested in CEF cultures as described in the preceding paragraph.

Reverse transcriptase assay

CEF culture fluids collected 72 hours after inoculation with the virus strain under study were clarified by centrifugation at 8000 *g* for 20 min. After further centrifugation of the supernatant at 116,000 *g* for 90 min in a SW-27 rotor of Beckman Spinco L2-65B ultracentrifuge the resulting virus pellet was homogenized in 200 μl 10 mM Tris-HCl + 0.1 M NaCl, pH 7.4, buffer and stored at −70 °C until used. Simultaneously processed fluids from uninoculated CEF cultures and from REV strain T-infected, REV-H-1 strain-infected or avian lymphoid leukosis virus strain HPRS-F42-infected CEF cultures served as controls for the assay. RNA-directed DNA polymerase (reverse transcriptase) activity was assayed in the standard exogenous reaction (Waite and Allen, 1975) in 20 μl final volume of a reaction mixture containing 50 mM Tris-HCl, pH 8.3, buffer, 50 mM KCl, 6 mM $MgCl_2$, 5 mM dithiothreitol, 0.2%

(v/v) Triton X-100, 0.5 mM thymidine triphosphate, 0.5 mM ^{3}H-thymidine triphosphate (specific activity 2.2 TBq/mmol, Amersham International plc, Aylesbury, England) and 1 μg poly(rA) $\times$ oligo(dT)15 template-primer (Boehringer Mannheim GmbH, Germany). Two to three μl samples were added to this reaction mixture and incubated at 37 °C for 30 min. The samples were then spotted onto Whatman DE-81 paper discs, treated as described by Maniatis et al. (1982) and counted for radioactivity in Bray's scintillation fluid with an LKB Rackbeta 3 liquid scintillation counter. Results are expressed as ^{3}H-thymidine uptake in counts per minute (cpm), unadjusted for background.

Electron microscopy

The cells from infected and control CEF cultures were fixed in 2.5% (v/v) glutaraldehyde, post-fixed in 1% (w/v) osmium tetroxide and embedded in Durcupan (Fluka AG, Buchs, Switzerland). Ultrathin sections were cut and stained with uranyl acetate–lead citrate and examined in a Jeol-100B electron microscope.

Assay for REV antibody

Turkey serum or plasma samples were tested for antibodies to REV by the indirect IF technique described elsewhere (Drén et al., 1988) using REV strain-T infected CEF cells as antigen. All samples were tested at 1 : 20 dilution.

Pathological studies

Dead and/or culled ailing turkeys were dissected, and portions of visceral organs with or without gross tumourous lesions as well as thymus, bursa of Fabricius and the nervous system were fixed in 10% (v/v) neutral formaldehyde and 5 μm sections were stained with haematoxylin and eosin. Culled and dead birds from pathogenicity experiments were processed likewise.

Test of humoral immune response

At 30 weeks of age, 12 turkeys from the 1-day-old inoculated groups and eight control turkeys were immunized intra-abdominally with 0.4 ml of 25% (v/v) sheep red blood cells (SRBC) and with about 10^{10} inactivated *Brucella abortus* organisms (Phylaxia Veterinary Biologicals Co., Budapest, Hungary) per kg of body mass, and boosted with the same antigen doses three weeks later. Sera were collected seven days after each immunization for determining primary and secondary antibody responses. Antibodies to SRBC and *B. abortus* were assayed on microtitration plates (Takátsy, 1955) in the presence or absence of 0.4 M 2-mercaptoethanol. Twofold dilutions of serum were tested,

and antibody titres were expressed as the $\log_2$ of the reciprocal of the highest serum dilution to demonstrate agglutinating activity. Arithmetic means $\pm$ standard error values for the experimental groups were calculated. Student's *t*-test was used for calculation of statistical significance.

Mitogen stimulation assay

Mitogen stimulation microassay with whole-blood cultures was performed on 96-well flat-bottomed microtitre plates (Microtest II, Falcon Plastics, Los Angeles, U.S.A.) according to a modification of the method described by Lee (1978). After 48 hours of incubation at 37 °C in a 5% (v/v) CO_2 humidified atmosphere of 10 μl heparinized whole blood in 200 μl RPMI 1640 medium (Flow Laboratories Ltd, Irvine, Scotland) with or without 5 μg/well Concanavalin A (Serva Feinbiochemica GmbH and Co., Heidelberg, Germany), 0.5 μCi of ^{3}H-thymidine (specific activity 185 GBq/mmol) was added to each well. After a 16-hour pulse with the radiolabel, the cells were harvested by a semiautomatic multiple culture cell harvester on Whatman GF/A glass filters. The filters with the deposited cells were washed five times with Dulbecco's PBS (pH 7.2), extracted with ice-cold 6% (w/v) trichloroacetic acid, washed once with methanol, dried and counted for radioactivity in Bray's scintillation fluid using a Packard liquid scintillation counter. Each sample was tested in six wells with or without mitogen. Results were expressed as mean values of cpm of the stimulated cultures and unstimulated control cultures, transformed to $\log_e$, and are given as total mean $\pm$ standard error per animal group. Statistical analysis of the $\log_e$ data was conducted by the Student's *t*-test.

Pathogenicity tests of REV isolates

The pathogenicity of the new REV isolates was studied in two separate experiments. In the first trial, $10^{3.5}$ 50% tissue culture infective dose/0.2 ml of cell-free supernatant fluid from the second CEF passage of field virus isolates designated REV-H-10 and REV-H-16 was inoculated via the yolk sac into two groups of 14-day-old turkey embryos which were hatched with parallel uninoculated controls. The poults hatched from inoculated and control eggs were housed in separate rooms. At 4 weeks of age a random sample was bled and examined for REV viraemia and antibodies to REV. Positive turkeys were bled again at 8 and 12 weeks of age for viraemia and antibody test. The experiment was terminated when the birds were 16 weeks old.

In the second trial, 71 1-day-old turkey poults were divided into five groups. The first two groups consisted of 17 and 14 turkeys which were inoculated intraperitoneally at one day of age with $10^{3.5}$ 50% tissue culture infective dose/0.2 ml of cell-free supernatant fluid from the second CEF passage of REV-H-10 and REV-H-16 isolates, respectively. The third group consisted



Fig. 1. Nodular tumours in the liver of a 56-week-old breeder turkey. The spleen is moderately enlarged and mottled

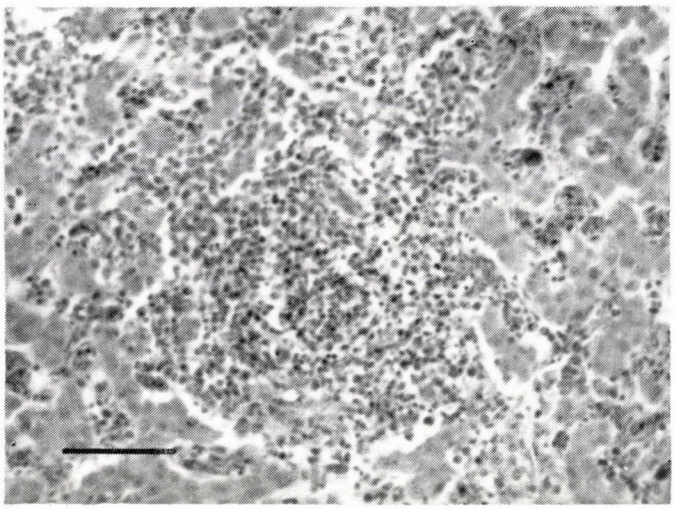


Fig. 2. Focal proliferation of tumour cells in the liver. Haematoxylin and eosin. Bar = 150 μm

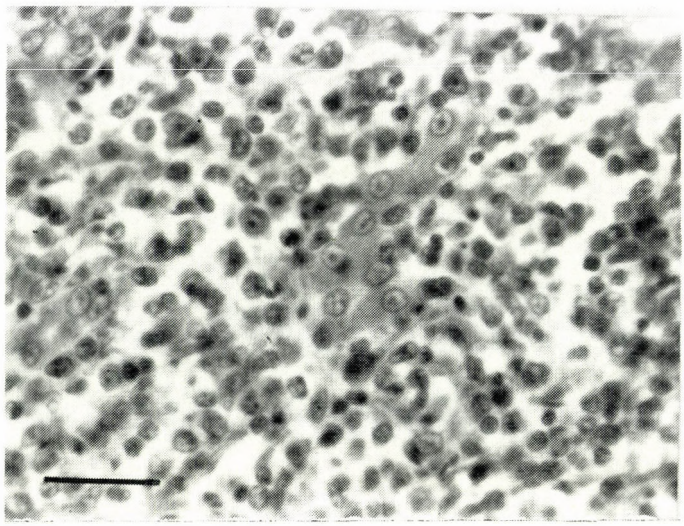


Fig. 3. Section of a lesion from a tumourous liver showing proliferation of moderately pleomorphic lymphoblastoid cells. Haematoxylin and eosin. Bar = 20 μm

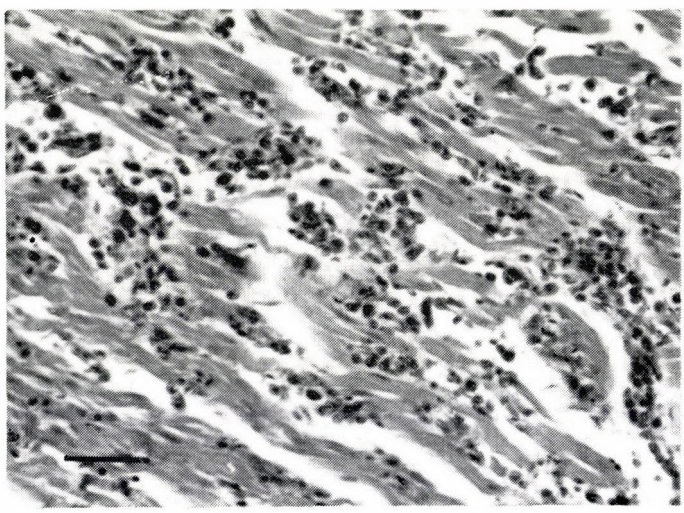


Fig. 4. Mononuclear cell infiltration of myocardium in a tumour-bearing adult turkey. Haematoxylin and eosin. Bar = 100 μm

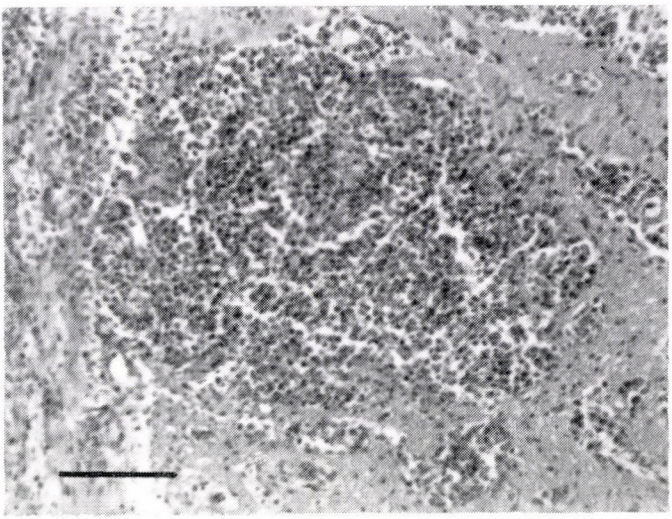


Fig. 5. Section of duodenum showing focal proliferation of lymphoblastoid cells in the mucosal layer. Haematoxylin and eosin. Bar = 150 μm

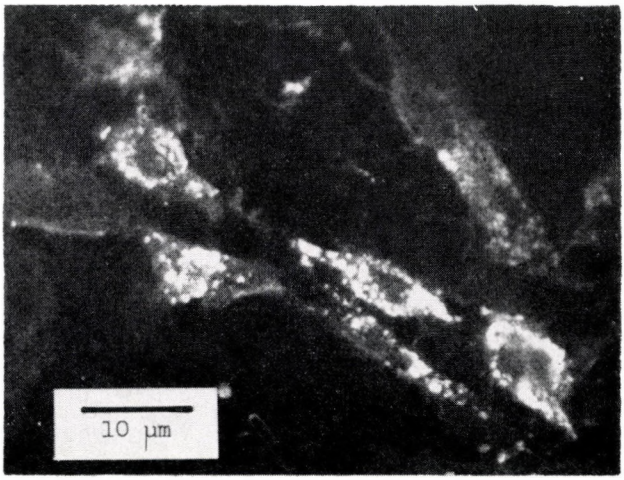


Fig. 6. Granular cytoplasmic immunofluorescence of a reticuloendotheliosis virus isolate on chick embryo fibroblasts. The cells were reacted with anti-REV-T chicken serum and stained with fluorescein-isothiocyanate-conjugated rabbit anti-chicken immunoglobulins



Fig. 7. Electron micrograph of chick embryo fibroblasts inoculated with plasma sample from an adult turkey with tumours showing C-type virus particles of about 100 nm in the extracellular space and budding from the plasma membrane. Bar = 200 nm

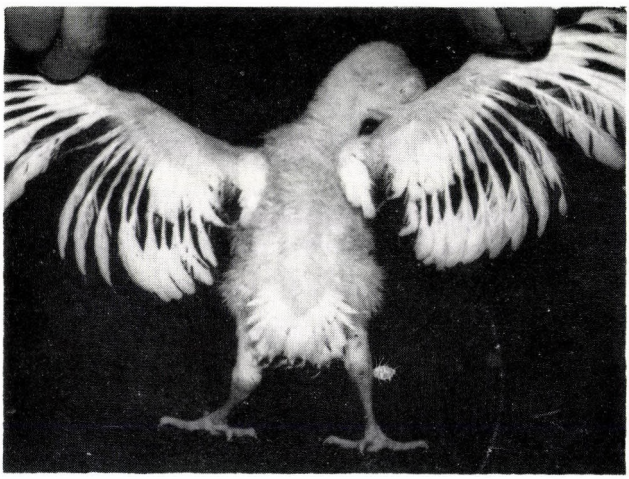


Fig. 8. Feathering defect of a turkey poult inoculated as 14-day embryo with $10^{3.5}$ 50% tissue culture infective dose of a reticuloendotheliosis virus field isolate. The barbs of the primary wing feathers either did not develop or stuck to the rachis on shorter or longer sections

of 10 poults which were kept in a separate room until 4 weeks of age, when they were inoculated intraperitoneally with the above dose of REV-H-16 isolate and then moved into the same room as the first two groups. The fourth group consisted of 18 turkeys which were contact exposed from one day of age by intermingling and housing together with the inoculated birds. The fifth group consisted of 12 turkeys which were kept separately to serve as isolated controls. Samples of serum and/or heparinized plasma were collected at 2, 4, 8 and 12 weeks of age for the determination of REV viraemia and antibodies to REV. At 30 weeks of age 12 of the surviving 1-day-old inoculated groups and eight of the control group were selected at random and bled for lymphocyte stimulation assays. They were bled again at 34 weeks of age for determination of the presence of REV viraemia and antibodies to REV. These turkeys were also immunized at 30 and 33 weeks of age with SRBC and *B. abortus* as described above to test humoral immune responsiveness. The experiment was terminated when the turkeys were 34 weeks old.

All birds dying during the experiments or killed at the end were necropsied, and the gross lesions were recorded. Some selected birds were examined histologically.

Results

Clinical features and pathology

The major health problem on the laying farm of an integrated turkey breeding enterprise was the high fluctuation of egg fertility among flocks. Egg infertility rate amounted to 64% in some flocks. A fowl pox like disease occurred sporadically on the farm during the laying period. Gross lesions were most prominent on the head skin and in the mouth, and occasionally were noted on the feathered skin of affected turkeys. Skin lesions consisted of raised red coalescent nodules, often with dry black necrotic surfaces, while the lesions in the mouth appeared as plaque-like caseous necrotic depositions under the tongue and at the commissures of the mouth. Total mortality of flocks due to enteritis, sporadic fowl pox like disease and visceral neoplasms varied between a few and about 10% during the production life. Mortality from lymphoid tumours was first noted around 20 weeks of age in some flocks, but usually occurred sporadically after 30 weeks of age. Total mortality from lymphoid tumours was estimated to be less than 2%.

Since REVs are known to cause cutaneous lesions reminiscent of fowl pox, a serological survey was conducted for the demonstration of the presence of REV infection in seven parent flocks experiencing sporadic fowl pox like lesions with or without considerable egg infertility. Results are given in Table I. In all flocks with more than 10% egg infertility, excepting P-J-1 flock, anti-

Table I

Incidence of antibodies to reticuloendotheliosis virus (REV) detected by the indirect immunofluorescence test in parent turkey flocks with or without candled egg infertility

Flock code	Infertility %	Turkeys tested for antibodies to REV				
		Age in weeks	Sex	No. tested	No. positive	% positive
P-J-1	50.2	44	M[a]	10	0	0
P-4	38.2	44	M	10	5	50
P-6	40.9	56	F[b]	50	12	24
P-7	11.7	28–45	M + F	97	30	30.9
P-J-2	4.0	44	F	20	0	0
P-3	8.3	36	M + F	48	1	2.1
P-8	5.9	53	M + F	35	5	14.3

[a] M = male
[b] F = female

bodies to REV were detected in 24 to 50% of random serum samples tested, whereas in those flocks with negligible egg infertility rate, the proportion of antibody positive samples ranged between nil and 14.3%.

When the farm was visited, necropsies were performed on all dead and sacrificed ailing turkeys between 28 and 56 weeks of age in those three flocks (P-4, P-6 and P-7) where the highest levels of REV seropositivity occurred. Seven birds were found to have gross neoplasms. Lymphoma-like nodular tumours were present regularly in the liver (Fig. 1) and occasionally in the pancreas, kidney and the wall of duodenum. Histological examination revealed focal and/or diffuse tumour cell proliferations and infiltrations in various visceral organs particularly in the liver (Fig. 2 and 3), spleen, heart (Fig. 4), pancreas and the wall of duodenum (Fig. 5). The proliferating tumour cells were relatively uniform large lymphoblastoid cells with moderate faintly basophylic cytoplasm and large vesicular nucleus containing one or two prominent nucleoli (Fig. 3). The nervous system and the bursa of Fabricius were normal by both gross and microscopic examination. Cutaneous lesions collected from other nontumourous birds in the same flocks proved to be of fowl pox origin.

Virus isolation and characterization

Virus isolations were made on plasma samples and cell suspensions of tumourous organs from adult turkeys in parent flocks where antibodies to REV had been demonstrated to occur at high frequencies. In 3 of 50 attempted isolations, a mild diffuse cytopathic effect appeared in CEF cultures about 7–8 days after inoculation. During the cytopathic effect the cell number decreased

somewhat in the infected cultures and most of their cells were more refractile and narrow than controls. On further incubation, the cells began to divide again until they produced a monolayer indistinguishable from the uninfected controls. In the cells of these cultures, a diffuse and/or granular bright-green cytoplasmic fluorescence occurred upon indirect IF staining with anti-REV-T chicken serum (Fig. 6). All the other inoculated and control cultures gave negative results by this test. Electron microscopic examination of ultrathin sections of the cells from the REV-positive cultures revealed virus particles of about 100 nm in diameter with C-type morphology in the extracellular space and budding from the cytoplasma membrane (Fig. 7). None of the cultures were positive for ALSV group-specific antigen by indirect IF staining with anti-AMV rabbit immune serum, while the cells of lymphoid leukosis virus strain HPRS-F42-infected CEF cultures, used as positive controls, gave prominent fluorescence.

Two virus isolates obtained from the plasma of a clinically normal turkey and from a naturally occurring lymphoma were selected for further characterization studies. These isolates were designated REV strain Hungary-10 (REV-H-10) and strain Hungary-16 (REV-H-16). Both isolates appeared to grow readily in CEF cultures with only a mild and transient cytopathology. The fluorescence in infected cells stained with the reference anti-REV-T chicken serum by the indirect IF technique was indistinguishable from that in nondefective REV strain T-infected cells. A similar cytoplasmic fluorescence was also observed when sera from convalescent turkeys experimentally inoculated with the new REV isolates were used for indirect IF staining of REV strain T-infected cells. Treatment by 5% (v/v) chloroform completely abolished infectivity of both isolates, indicating that they have a lipoprotein envelope. CEF

Table II

Reverse transcriptase activity of virus strains after concentration by ultracentrifugation from supernatants of infected chick embryo fibroblast (CEF) cultures

Virus strains	No. of passages in CEF	Enzyme activity (cpm)[a]
REV-H-10	3	15 670.1
REV-H-16	3	14 880.5
REV-T	3	26 892.0
REV-H-1	4	26 456.1
HPRS-F42[b]	6	977.8
None	2	42.0

[a]: RNA-dependent DNA polymerase enzyme activity as measured in the standard exogenous reaction in the presence of 6 mM $MgCl_2$ using poly(rA)×oligo(dT)15 template-primer. ^{3}H-thymidine uptake in mean counts per minute, unadjusted for background;
[b]: HPRS-F42 strain of avian lymphoid leukosis virus

Table III

Response of 14-day-old turkey embryos to infection with reticuloendotheliosis virus (REV) isolates

Inoculum[a]	Number of embryos		Antibody to REV[b]	Isolation of REV[c]
	inoculated	hatched		
REV-H-10	14	11 78.5%	1/9 11.1%	1/5 20%
REV-H-16	14	11 78.5%	0/6	1/5 20%
None	17	15 88.2%	0/8	0/3

[a]: $10^{3.5}$ 50% tissue culture infective dose per embryo of each virus as cell-free supernatant fluid from the second passage in chick embryo fibroblasts; [b]: Detected by the indirect immunofluorescence test in sera of 4-week-old turkeys. No. poults positive/No. tested; [c]: Assay on plasma samples from 4-week-old turkeys. No. poults positive/No. tested

propagated, concentrated and disrupted virus isolates showed high level of reverse transcriptase activity comparable to that of REV-T and REV-H-1 strains (Table II) used as positive controls.

Pathogenicity of REV isolates

The pathogenicity of the new REV isolates was studied in two experiments. In the first experiment, groups of 14-day-old turkey embryos were inoculated with each cell-culture-propagated virus isolate. As shown in Table III, about 10% less turkey poults hatched from REV-inoculated eggs than from parallel uninoculated controls. There was no difference between the two virus isolates. Feathering defects similar to 'nakanuke' (Koyama et al., 1976) were observed by 4 weeks of age in 2 of 22 poults hatched from inoculated eggs (Fig. 8). One of 15 poults had antibodies to REV and 2 of 10 poults were viraemic when tested at 4 weeks of age. The two viraemic turkeys were still viraemic and free of antibodies to REV at 12 weeks of age, and one of them died a week later with lymphomas in the liver, spleen, small intestines, proventriculus, pancreas and the bursa of Fabricius. Histologically, the lymphomatous lesions were similar in cytology to those seen in the naturally diseased turkeys. The other turkey was also found to be viraemic when it was killed at 16 weeks of age. There was no additional tumour mortality, and none of the survivors had gross neoplastic lesions when necropsied at 16 weeks of age. Control turkeys were clinically normal and they showed no lesions at termination. Also, neither REV nor antibodies to it could be demonstrated in the examined birds at 4 weeks of age.

In the second experiment, groups of turkey poults were infected with REV at 1 day of age, 4 weeks of age and by contact exposure from 1 day of age. Table IV shows the overall mortalities together with the tumour mortality for the various experimental groups. Twelve of 31 turkeys inoculated at 1-day-old died or were killed when moribund between 117 and 237 days of age, of these five showed gross lymphoproliferative lesions at autopsy. No mortality was noted in the 4-week-old inoculated group. Four turkeys died in the contact group at 122 days of age, and at necropsy one was found to have lymphoid tumours. This was the earliest tumour-associated death in the course of the experiment. Nonneoplastic losses in the control, contact and 1-day-old inoculated groups varied between 16.7 and 28.6%, and were mostly due to perosis, arthritis and occasionally to enteritis, chronic perihepatitis and airsacculitis. Survivors of the various experimental groups had no grossly detectable tumours when killed at the end of the experiment. The gross tumourous lesions found in the six affected turkeys were mainly nodular and occurred predominantly in the liver, spleen and duodenum but were also noted in the bursa of Fabricius in one bird. Histological examination confirmed the presence of diffuse and/or focal proliferations of moderately pleomorphic lymphoblastoid cells in the affected organs similar to the lesions observed in the natural disease. No cutaneous lesions occurred in affected turkeys, and peripheral nerves were normal by both gross and microscopic examination.

Cellular suspensions from tumour-bearing turkeys were used to infect CEF cultures. At the end of the 17-day cultivation period, the cultures infected

Table IV

Mortality and incidence of lymphoid tumours in groups of turkeys experimentally infected with reticuloendotheliosis virus (REV) isolates during a 34-week observation period

Group	Inoculum[a]	No. turkeys	Total mortality	No. with tumours		Age at death (range in days)	Isolation of REV[b]
				Dead	Survivors at 34 wks		
Inoculated at 1-day-old	REV-H-10	17	6 35.3%	3 17.6%	0	204–237	2/3
	REV-H-16	14	6 42.9%	2 14.3%	0	117–131	1/2
Inoculated at 4 weeks	REV-H-16	10	0	0	0		
Contact exposed		18	4 22.2%	1 5.6%	0	112	0/1
Isolated controls	None	12	3 25%	0	0	10–128	0/3

[a]: $10^{3.5}$ 50% tissue culture infective dose per poult of each virus as cell-free supernatant fluid from the second passage in chick embryo fibroblasts;

[b]: Assay on tumour tissue or spleen cells from dead tumour-bearing and control turkeys

with samples from three of six birds reacted specifically in the indirect IF test with the reference anti-REV-T chicken serum. A diffuse or granular cytoplasmic fluorescenc similar to that seen in cultures infected with field material was present. Tissues from all tumour-bearing turkeys were negative for ALSV by the same cell-culture assay using the indirect IF staining of infected cultures with anti-AMV rabbit serum.

Table V and VI present the incidence of REV viraemia, as detected by virus in plasma samples, and the development of antibody against REV in all experimental groups up to 12 weeks of age, respectively.

Table V

Incidence of reticuloendotheliosis virus (REV) viraemia in groups of experimentally infected turkeys

Group	Inoculum[a]	Age in weeks			
		2	4	8	12
Inoculated at 1-day-old	REV-H-10	0/17[b]	7/17 41.2%	5/14 35.7%	3/15 20%
	REV-H-16	0/14	6/14 42.8%	5/14 35.7%	3/11 27.3%
Inoculated at 4 weeks	REV-H-16	0/10	0/10	2/9 22.2%	1/7 14.3%
Contact exposed		0/18	0/15	8/14 57.1%	0/14
Isolated controls	None	0/10	0/9	0/7	0/7

[a]: $10^{3.5}$ 50% tissue culture infective dose per poult of each virus as cell-free supernatant fluid from the second passage in chick embryo fibroblasts;
[b]: Number positive over number tested

Table VI

Incidence of antibodies to reticuloendotheliosis virus (REV) detected by the indirect immunofluorescence test in groups of experimentally infected turkeys

Group	Inoculum[a]	Age in weeks			
		2	4	8	12
Inoculated at 1-day-old	REV-H-10	4/17[b] 23.2%	6/17 35.3%	5/15 33.3%	5/15 33.3%
	REV-H-16	3/14 21.4%	5/14 35.7%	5/14 35.7%	2/11 18.2%
Inoculated at 4 weeks	REV-H-16	0/10	0/10	8/9 88.9%	5/7 71.4%
Contact exposed		0/18	0/15	2/14 14.3%	10/14 71.4%
Isolated controls	None	0/10	0/9	0/7	0/7

[a]: $10^{3.5}$ 50% tissue culture infective dose per poult of each virus as cell-free supernatant fluid from the second passage in chick embryo fibroblasts;
[b]: Number positive over number tested

Table VII

Immune responsiveness of adult turkeys experimentally infected at 1-day-old with a reticuloendotheliosis virus field isolate

Group	Antibody response to immunization with				Con-A response (log_e cpm)[c]
	SRBC[f]		*Brucella abortus*		
	primary 31 wks	secondary 34 wks	primary 31 wks	secondary 34 wks	30 wks
Inoculated	[a]5.0 ± 0.4	4.3 ± 0.2[d]	5.0 ± 0.2	2.3 ± 0.1[d]	8.62 ± 0.1[e]
n = 12	[b]1.3 ± 0.4	1.6 ± 0.2	0.7 ± 0.1	0.8 ± 0.2	
Control	[a]6.2 ± 0.5	6.3 ± 0.4	5.1 ± 0.2	5.3 ± 0.4	9.50 ± 0.1
n = 8	[b]0.8 ± 0.6	2.2 ± 0.3	0.2 ± 0.1	0.7 ± 0.2	

[a]: Mean ± standard error of log_2 agglutination titres of sera prior to 2-mercaptoethanol treatment; [b]: Mean ± standard error of log_2 agglutination titres of sera after 2-mercaptoethanol treatment; [c]: Mean ± standard error log_e counts per minute of ^{3}H-thymidine uptake by Concanavalin A stimulated lymphocytes; [d]: Significant at $P < 0.05$ level (compared with control); [e]: Significant at $P < 0.001$ level (compared with control); [f]: SRBC = sheep red blood cells

Control turkeys were clinically normal throughout the 34-week observation period. There was no REV-related mortality in the group, and all survivors remained free of REV infection based on serological tests and virus isolation attempts from plasma samples.

The effects of REV infection on immunocompetence are reported in Table VII. Those 12 turkeys of the 1-day-old inoculated groups that were selected at random for immunoresponsiveness studies were nonviraemic and only 5 of them were positive for REV antibody at 30 and 34 weeks of age. These birds were partially immunosuppressed. Their blood lymphocytes were substantially less responsive to stimulation with Concanavalin A than were those of uninoculated control turkeys. Statistical analysis indicated no significant difference between the log_e counts incorporated by nonstimulated lymphocytes from birds of the two experimental groups (data not shown in Table VII). REV-inoculated turkeys were not deficient in the primary antibody response to SRBC and *Brucella abortus* antigens. Their antibody titres following the secondary immunization were, however, significantly lower than those of controls. There was no statistically significant difference between the titres of 2-mercaptoethanol resistant antibodies to either antigen.

Discussion

Thus far, REV has seldom been isolated from turkeys (McDougall et al., 1978; Paul et al., 1976; Sarma et al., 1975; Solomon et al., 1976; Robinson and Twiehaus, 1974; Witter and Glass, 1984). In the present study natural

REV infection was demonstrated, by both serology and virus isolation, in breeding turkey flocks experiencing low levels of mortality from leukotic tumours, varying degree of egg infertility and sporadic cutaneous lesions reminiscent of fowl pox.

The pathology of neoplasms was similar to natural reticuloendotheliosis outbreaks in turkeys described by others (McDougall et al., 1978; Paul et al., 1976; Sarma et al., 1975; Solomon et al., 1976; Witter and Glass, 1984). However, in accordance with previous findings (Witter and Glass, 1984), we also observed no enteritis and bursal tumours, which were reported by McDougall et al. (1978), and the peripheral nerves lacked mononuclear endoneural infiltrations described by others (Hanson and Howell, 1979; Paul et al., 1976). Histopathological examinations have revealed that the skin lesions observed in the present case were of fowl pox origin and not a cutaneous manifestation of reticuloendotheliosis as reported by Hanson and Howell (1979).

The major production problem of the investigated laying farm was the fluctuation of egg infertility between 4.0 and 64% during the laying cycle. Fertility problems have not yet been reported to occur in association with natural REV infection in turkeys (Witter and Glass, 1984). Although the frequency of REV antibody was generally higher in those flocks of the farm where high levels of infertility occurred, it could not be concluded from the data reported here that infertility was a direct consequence of REV infection, because the number of tested birds was relatively low (altogether 167 turkeys from four flocks with an infertility rate above 10% and 103 turkeys from three problem-free flocks), and because there were flocks where considerable infertility (50.2%) occurred in the absence of REV infection (P-J-1 flock, Table I) and, conversely, where high incidence of REV antibody (30.9%) was associated with relatively low (11.7%) egg infertility rate (P-7 flock, Table I). Coincidence of high infertility rate with high REV seropositivity in two flocks (P-4 and P-6 flocks, Table I) suggests, however, a possible contributory role of REV infection. Thus, clarification of this point deserves further studies.

In tissue cultures, chloroform sensitive viruses of about 100 nm in diameter with C-type morphology and RNA-directed DNA polymerase activity were isolated from plasma samples of clinically healthy turkeys and from selected lymphoid tumours of turkeys over 28 weeks of age. The virus isolates were similar to the prototype strain T REV in cultural, morphological and antigenical characteristics. This indicates that they are members of the REV group of avian retroviruses. The virus stocks were determined to be free of contamination with exogenous ALSVs by cell-culture assay using the indirect IF staining with rabbit anti-AMV immune serum for the detection of ALSV group-specific antigen (Payne et al., 1966).

Results of pathogenicity studies showed that in embryonally, neonatally and contact infected turkeys, REV field strains produced a chronic neoplastic

disease similar in gross and microscopic appearance to that seen in the natural cases of the disease, with latent periods of 91 to 237 days. Some of the hatched poults inoculated as 14-day embryos developed feathering defects similar to 'nakanuke' seen in experimental REV infections in chickens (Koyama et al., 1976; Motha and Egerton, 1987). As expected from other studies (McDougall et al., 1980; Purchase et al., 1973; Witter and Johnson, 1985), the REV field strains were more oncogenic when exposure was by inoculation than when exposure was by contact. Four-week-old inoculated turkeys did not develop visible lymphomas, but less susceptibility of older birds has been noted previously (McDougall et al., 1980). In accordance with the report of McDougall et al. (1978), we also observed bursal tumours in two experimentally infected turkeys that died at 91 and 231 days of age. Whether these tumours are comparable to the B-cell neoplasms caused by other nondefective strains of REV in experimentally infected chickens (Witter and Crittenden, 1979; Witter et al., 1981) and are induced by a similar molecular mechanism (Noori-Daloii et al. 1981) remains to be elucidated. Horizontal spread of REV infection has been reported to occur in chickens, ducks and turkeys (McDougall et al., 1980; Paul et al., 1977; Purchase et al., 1973; Witter and Johnson, 1985). In this study the virus was also found to spread readily among turkeys as evidenced by the presence of viraemia and serum antibodies and the development of tumours in turkeys held in direct contact with others inoculated with field isolates of the virus shortly after hatching.

During the pathogenicity experiments, REV with the same features as the field isolates was reisolated from 57% of tumour-bearing turkeys. REV viraemia and serum antibodies to REV were also detected from a number of the survivors in all experimentally infected groups. In view of previous findings (McDougall et al., 1980), the poor induction of tolerant infection, i.e. persistent viraemia in the absence of serum antibodies, by embryonal or neonatal inoculation, as reported here, was somewhat unexpected. However, it has been demonstrated in chickens (Witter et al., 1981) that induction of tolerant infection depends not only on the age at infection but also on the strain and dose of virus. Thus, the induction of lower rates of tolerant infection in our studies could be due to the differences in the strains of REV and to the lower input virus doses used for inoculation. A differential susceptibility between the breeds of turkeys used for the experiments may also play a role.

The effect of REV infection on the immune competence of turkeys has not yet been studied. In this study neonatally inoculated turkeys that developed apparent nontolerant infection, defined here as the presence of detectable immunofluorescent serum antibodies, were found to be deficient in secondary antibody response to SRBC and *B. abortus* antigens and in mitogen-induced blastogenesis of peripheral blood lymphocytes at 30 to 34 weeks of age. This observation is noteworthy, since the long-term immunosuppressive potential

of nondefective REVs in turkeys has not been reported previously. The mechanism by which immunosuppression occurs in REV-infected turkeys is not clear. Further work is therefore required to clarify whether this unusual manifestation of immunosuppression was a peculiarity of our virus isolates or was due to a different host response in that nontolerant REV infection in turkeys, unlike in chickens (Carpenter et al., 1977, 1978; Rup et al., 1979; Walker et al., 1983; Witter and Johnson, 1985; Witter et al., 1981), leads to the development of a long-lasting immunosuppressed state.

The results in the present study fulfil Koch's postulates. We are therefore entitled to suggest that the naturally occurring neoplastic disease of turkeys described in this paper was caused by new strains of REV. Cultural and pathogenicity characteristics suggest that these viruses are similar to the nondefective helper REV (Hoelzer et al., 1979). Their origin is unknown.

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STUDIES ON THE VIRULENCE OF PIGEON PARAMYXOVIRUS-1 (PMV-1) I. CHANGES IN THE VIRULENCE OF PIGEON PMV-1 STRAINS ISOLATED IN HUNGARY UPON PASSAGE IN CHICKENS, EMBRYONATED HEN'S EGGS AND PIGEONS

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Pigeon paramyxovirus 1 (PMV-1) strains isolated in Hungary were examined for latent virulence to chickens. They were serially inoculated in chickens and their virulence was measured after the fifth passage. Three out of eight strains showed an increased virulence (IVPI = 1.3–1.7) after five passages in chickens. A transition of virulence from the mesogenic to velogenic category was not observed. Passage of known mesogenic laboratory strains (like vaccine strain H, Mukteswar, etc.) in chickens was only partially successful. Only the virulence of strain Roakin could be increased by passaging it in chickens (IVPI rose from 0.4. to 1.6). The same three PMV-1 strains exhibited a slight increase in virulence after ten passages in chicken embryos. Two strains whose virulence did not increase either in chickens or in eggs (IVPI = 0) showed some increase in virulence when they were passaged in pigeons (IVPI = 0.6–1.0). Thus the pigeon might be one of the hosts where virus heterogeneity is generated in nature.

Keywords: Pigeon paramyxovirus-1 (PMV-1), virulence, change, passage.

The susceptibility of pigeons to fowl pest, a disease known as Newcastle disease since an outbreak in Great Britain in 1926, has been known for a long time (Lode and Gruber, 1901; Centanni, 1902; Halász, 1912). These early works had reported nervous signs and enteritis in pigeons. Subsequently several authors reported field outbreaks among pigeons (Picard, 1928; Hanson and Sinha, 1952; Ulbrich and Sodan, 1965; Hilbrich, 1972; Stewart, 1971; Vindevogel et al., 1972; Utterback and Schwartz, 1973; Maes et al., 1974; Pearson and McCann, 1975; Canic, 1981). The common features of the above listed cases were *(i)* the lack of evidence for direct spread of infection between pigeon flocks (i. e. spread without the involvement of chickens); *(ii)* that all outbreaks had occurred in pigeons living in proximity to chickens affected by ND; and *(iii)* that the ND virus (NDV) strains isolated from those outbreaks were velogenic for chickens (Hanson, 1975).

In 1981 an ND-like disease, spreading from pigeon to pigeon, emerged in Europe. The disease was first described in Italy (Biancifiori and Fioroni, 1983), then it appeared in several countries of Western Europe (Viaene et al.,

1983; Richter, 1983; Kaleta et al., 1984; Guittet et al., 1984; Alexander et al., 1984*a* and *b)* and in Hungary (Povazsán et al., 1984; Sólyom, 1984).

The ND-like disease spreading among pigeons was caused by a paramyxovirus type 1 (PMV-1) virus. This agent showed high levels of cross-reaction to NDV in the haemagglutination inhibition (HI) test but could unambiguously be differentiated from NDV strains by monoclonal antibodies (Alexander et al., 1984).

Attempts at infecting chickens with the pigeon PMV-1 strains succeeded. Most of the pigeon PMV-1 strains, however, proved mesogenic for chickens (Alexander and Parsons, 1984). The virulence of some strains for chickens was markedly increased by passage through chickens (Alexander and Parsons, 1984). This latent virulence for chickens may have contributed to the severe ND outbreaks caused by pigeon PMV-1 strains in chicken flocks not immunized against ND in Great Britain (Alexander et al., 1984*b)*.

To assess the risks constituted by pigeon PMV-1 strains to chicken flocks, the biological properties of pigeon PMV-1 strains isolated in Hungary were examined. It was found that *(i)* most of the Hungarian pigeon PMV-1 isolates belonged to the mesogenic virulence group (Kissi et al., 1988); *(ii)* the virulence of several mesogenic pigeon PMV-1 strains isolated in Hungary was significantly increased by passage through chickens (this paper); and *(iii)* the virulence increase occurring during passage through chickens resulted from selection (see the accompanying paper, Kissi and Lomniczi, 1988).

Materials and methods

Virus strains

The pigeon PMV-1 strains were obtained from the Central Veterinary Institute (Budapest) by the courtesy of Dr. E. Sághy. Strains 61, 201, 208 and 225 were kindly provided by Dr. J. Tanyi (Veterinary Institute, Debrecen).

For the origin of the pigeon PMV-1 and mesogenic NDV strains, see earlier papers (Kissi et al., 1988; Russel and Alexander, 1983).

Virus propagation

The virus strains were grown in the allantoic cavity of 9- to 11-day-old chicken embryos (Lomniczi, 1973). The same procedure was followed when reisolating the virus from 20% suspensions of chicken organs (liver, spleen, or brain).

Cell cultures, plaque purification and plaque isolation

These were performed as described earlier (Lomniczi, 1973 and 1974).

Intravenous pathogenicity index (IVPI) tests

The IVPI tests were done in SPF chicks by a standard procedure(Anon., 1963). IVPI was calculated after 12 days.

Virus passage through chickens

The chickens were inoculated intramuscularly with a 20% liver-spleen suspension. Simultaneously with injecting chickens with the organ homogenates, embryonated eggs were inoculated to determine the virus concentration of the suspensions.

Results

Changes in the virulence of pigeon PMV-1 strains upon passage through chickens

The virulence of 33 out of the pigeon PMV-1 strains isolated in Hungary was determined in six-week-old SPF chickens. Thirty-two out of the 33 isolates were assigned to the mesogenic virulence category (Kissi et al., 1988).

As British researchers reported a considerable increase in the virulence of some pigeon PMV-1 strains upon passage through chickens, it was of interest to examine whether or not this was true for some strains isolated in Hungary and continental Europe. Eight Hungarian strains and four strains isolated

Table I

Virus titres of the 20% liver-spleen homogenate in the different passages (the initial inoculum contained 10^6 PFU/chicken)

PMV-1 strains	Plaque-forming units/0.1 ml in passage				
	1	2	3	4	5
48	0*	0	3	130	>300
52	0	37	115	38	>150
55	0	1	4	79	>300
56	4	0	110	240	>130
57	0	28	0	33	>100
58	0	23	103	150	>220
61	0	0	0	0	10
234	0				
209	0	0	0	80	>200
218	0	0	0	5	7
227	3	5	7	10	>200
266	0	5	1	0	4

* If the plaque count was 0, the virus was always demonstrable by inoculating fertile eggs. Furthermore, in most cases it could be passaged without previous enrichment in eggs

Table II

Effect of passage through chickens on the virulence of pigeon PMV-1 strains

Virus strain	Origin	Virulence (IVPI)	
		Initial inoculum	5th passage
48	Hungary, 1983	0.0	0.0
52	Hungary, 1983	0.66	0.72
55	Hungary, 1983	0.0	1.30
56	Hungary, 1983	0.18	1.34
57	Hungary, 1983	0.0	0.0
58	Hungary, 1983	0.0	1.68
61	Hungary, 1983	0.0	0.0
234	Hungary, 1983	0.6	1.8
209	The Netherlands, 1983	0.36	0.10
218	Iraq, 1978	0.40	0.40
227	Italy, 1982	0.0	0.0
266	Yugoslavia, 1984	0.0	0.0

outside Hungary were passaged five times through chickens at 4- to 5-day intervals. None of the chickens became ill. By inoculating embryonated hen's eggs, the virus was always detectable in the liver and spleen homogenate used for passage.

The virus titre of the organs permitted successful passage of the virus with the 20% organ homogenate itself. Previous virus propagation in eggs was required only exceptionally (sometimes after the first passage). Table I shows that parallel to the rise of the number of passages virus titres in the liver and spleen increased.

After the 5th passage in chickens, IVPI tests were done in 6-week-old chickens with the initial inoculum and with virus from the 5th passage, after previous enrichment in embryonated eggs (Table II). The virulence of strains designated 55, 56 and 58 increased considerably (IVPI = 1.3–1.7), while that of the other strains did not change. Virus could always be recovered from the brain of moribund or dead chickens.

Changes in the virulence of mesogenic NDV strains upon passage through chickens

To judge the significance of virulence increase it would be important to know whether this was a property of mesogenic NDV strains (including numerous vaccine strains) in general, or was only an indicator of the latent chicken pathogenicity of pigeon PMV-1 strains. Namely, it is possible that the virulence of the mesogenic strains known by us is not stable; since, however, no

attempts have been made to alter it experimentally (e. g. by passage through chickens), this phenomenon has not been observed in connection with these strains so far.

Although the above phenomenon has obvious practical bearings, there are no data in the literature on how mesogenic NDV strains, viruses closest in virulence to the pigeon PMV-1 strains, behave under similar conditions. Therefore, some mesogenic NDV isolates long known from the literature and some recent field isolates (strains 201 and 208), of this group were passaged through chickens. The passage and virulence of these strains are shown in Table III. Some of the strains proved non-passageable in chickens. Only strain *Roakin* could be maintained up to the 5th passage, the others "died out" earlier, similarly to strain 225, a lentogenic field isolate which had been expected to be non-passageable at all. (Some strains could be passaged only after they had been grown up in embryonated hen's eggs between the passages.) Strain *Roakin* was not only passageable but also underwent a considerable virulence increase, whereas the virulence of the other strains failed to increase.

Changes in the virulence of pigeon PMV-1 strains upon passage in embryonated hen's eggs

The above experiments have shown that though in pathogenicity to chickens the pigeon PMV-1 strains are similar to the mesogenic NDV strains (e. g. IVPI $\leq$ 1.5), the former not only grow well in chickens but during pas-

Table III

Passageability of mesogenic NDV strains through chickens (chickens in the first passage received 10^6 PFU/bird)

Virus strain	Origin	Virulence (IVPI)	
		Initial inoculum	Last passage
H	Great Britain, 1933	0.33	0.28 (2nd)
H/S3	(Selected from H)*	0.0	n.p.
L	USA, 1940s	0.38	0.0 (4th)
Komarov	Palestine, 1940s	0.0	0.5 (5th)
TCND	USA	0.0	n.p.
Beaudette C	USA, 1946 (1959)	0.0	n.p.
Mukteswar	India, 1940s	0.0	0.0 (3rd)
Roakin	USA, 1949	0.4	1.6 (5th)
201	Hungary, 1980	0.50	0.0 (3rd)
208	Hungary, 1981	0.40	0.23 (3rd)
225	Hungary, 1984	0.0	n.p.

* Prokopovitsch and Lomniczi, 1973; n.p. = non-passageable

Table IV

Changes in the virulence of pigeon PMV-1 strains upon passage in fertile hen's eggs

Virus strain	Virulence (IVPI)		
	Initial inoculum	5th passage	10th passage
48	0.0	NT	0.0
55	0.0	0.0	0.28
56	0.34	NT	0.54
57	0.0	NT	0.0
58	0.0	0.23	0.41
61	0.0	NT	0.0
227	0.0	NT	0.0
266	0.0	NT	0.0

NT = not tested

sage their virulence may increase. Before starting investigations into the mechanism of the change of virulence, the behaviour of these viruses during passage in embryonated hen's eggs was examined.

Pigeon PMV-1 strains of changing and those of unchanging virulence were passaged ten times in embryonated hens' eggs, then IVPI tests were done on the initial inoculum and the 10th passage (Table IV). A slight increase in virulence was found for strains 55, 56 and 58.

Changes in the virulence of pigeon PMV-1 strains upon passage through pigeons

The experimental data shown above raise another problem: if some of the pigeon PMV-1 strains are composed of heterogeneous virus populations (see also the accompanying paper, Kissi and Lomniczi, 1988), how does this heterogeneity arise? As the natural host of pigeon PMV-1 strains is the pigeon, the question has arisen whether or not multiplication of the virus in pigeons favours the emergence of this heterogeneity. Therefore, three pigeon PMV-1 strains were selected whose virulence had not changed (i. e. remained 0) upon

Table V

Changes in the virulence of pigeon PMV-1 strains upon passage in pigeons

Virus strain	Initial inoculum	Virulence (IVPI) 5th passage	
		chicken	pigeon
48	0.0	0.0	1.0
57	0.0	0.0	0.0
227	0.0	0.0	0.6

passage through chickens. These strains were passaged through pigeons (Table V). The virulence of two out of the three isolates (strains 48 and 227) has somewhat increased.

Discussion

The aim of the present investigations was to assess the threat constituted to domestic poultry flocks by virus strains that spread in Hungary during outbreaks in pigeons.

At the start of these investigations, data available in the literature were restricted to the virulence of pigeon PMV-1 strains isolated in Great Britain (Alexander and Parsons, 1984). According to these data, most pigeon PMV-1 strains were similar in virulence to mesogenic NDV strains, and some of them were more virulent than the others. It is easy to realize that pigeons carrying NDV varieties of such virulence are a potential threat as virus transmitters to chicken flocks. There are two additional properties which underline the risks connected with such mesogenic strains: *(i)* from pigeons infected with pigeon PMV-1 the virus was easily transmitted to chickens placed among them; and *(ii)* the virulence of the virus strains was increased experimentally by passage through chickens (Alexander and Parsons, 1984). Even with knowledge of these facts, it came as a surprise when in mid-1983 the virus appeared in more than 20 susceptible chicken flocks of Great Britain (Alexander et al., 1985). The causative agent of these outbreaks was identified as a pigeon PMV-1 strain by monoclonal antibodies.

In the light of these facts it was clear that in order to assess the possibility of similar outbreaks in Hungary and draw an analogy with events in Great Britain, the Hungarian PMV-1 isolates had to be studied in detail.

The virulence of most pigeon PMV-1 strains isolated in Hungary is similar to that of the mesogenic NDV strains (Kissi et al., 1988), similarly to what was found in Great Britain. Nevertheless, the virulence of four out of eight pigeon PMV-1 strains selected at random has increased significantly upon passage through chickens. (One of these strains, a mesogenic isolate, had come from a chicken flock where it caused no outbreak). This latent virulence of pigeon PMV-1 strains has been confirmed with strains isolated in The Netherlands (Bijnens and Devos, 1987). On the other hand, out of ten mesogenic NDV strains only strain *Roakin* showed a similar phenomenon. The passageability of these latter strains was poor: in most cases passage was successful only if the viruses were grown in embryonated hen's eggs between the individual passages. Some strains could not be passaged at all.

Another property in which pigeon PMV-1 strains constitute a greater epizootiological hazard than do mesogenic NDV strains, is their increased contagiosity to chickens. In the present experiments, in four-day-old SPF

chickens contact infected with strains 52 and 56 the infection rate was 100% and the death rate was between 50 and 60%. A similar contagiosity was reported by others (Bijnens et al., 1985).

Upon passage through embryonated hen's eggs the same three strains (55, 56 and 58) underwent a slight increase in virulence as in chickens, though in the case of eggs this virulence increase was demonstrable only after the 10th passage. Selection of the more virulent virus population (see the accompanying paper, Kissi and Lomniczi, 1988) takes place later during passage in eggs.

The experiments described above have indicated that during passage through chickens more virulent lines are selected from a presumably heterogeneous virus population. Consequently, some pigeon PMV-1 strains are more heterogeneous in respect of virulence than others. It would be important to elucidate what factors participate in inducing this heterogeneity. It is possible that multiplication of the virus in pigeons favours the development of a heterogeneous virus population. Therefore, pigeon PMV-1 strains exhibiting the lowest degree of heterogeneity (i.e. the virulence of which failed to increase upon passage through either chickens or embryonated hen's eggs) were passaged in pigeons. Two strains (48 and 227) became heterogeneous after passage through pigeons, as indicated by increase of their virulence to chickens.

Besides Great Britain, pigeon PMV-1 strains have been reported to cause outbreaks among chickens in Austria too (Vasicek and Schusser, 1985). At the time of the outbreaks no immunization against ND was practised in either country.

Although a pigeon PMV-1 strain of mesogenic virulence was isolated from a chicken flock in 1983, no outbreak connected with pigeon PMV-1 has been observed in Hungary.

As some of the Hungarian pigeon PMV-1 isolates share the virulence characteristics (high contagiosity to chicken and tendency for virulence increase) of strains isolated in countries where they did cause outbreaks in chickens, one may speculate whether the absence of such outbreaks in Hungary can be attributed, at least in part, to the fact that most of the chicken flocks here are immunized against ND. This was one of the most important differences between the situation in Hungary and that prevailing at the time in Great Britain and Austria. It is also questionable whether or not a mesogenic PMV-1 strain of "raised virulence" can start an outbreak even among susceptible chickens. It is to be noted that the virulence increase of no pigeon PMV-1 strain examined here reached the velogenic level. It is more likely that in Great Britain and Austria strains velogenic for chicken, i. e. more virulent than the majority of the mesogenic pigeon PMV-1 strains, caused the outbreaks, since their rare occurrence may have been counterbalanced by the complete susceptibility of the chicken flocks. In Hungary there are much few-

er susceptible chicken flocks, and pigeon PMV-1 strain of very high virulence was introduced only on one occasion (Kissi et al., 1988).

In the future the preventive vaccination of pigeons on a large scale will further reduce the risk of chicken outbreaks by decreasing the number of outbreaks occurring among pigeons. Nevertheless, pigeons infected by pigeon PMV-1 strains should be considered a potential source of infection as long as the disease is not eradicated.

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STUDIES ON THE VIRULENCE OF PIGEON PARAMYXOVIRUS-1 (PMV-1) II. MECHANISM OF THE VIRULENCE INCREASE OF PIGEON PMV-1

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The virulence of individual plaques isolated from a pigeon PMV-1 strain was determined in 6-week-old chickens. It was shown that PMV-1 strains are heterogeneous in virulence. Pigeon PMV-1 strains (IVPI = 0.0) that exhibited a virulence increase during passages in chickens (IVPI = 1.3–1.8) yielded plaques whose virulence was elevated (IVPI = 0.9–1.2) though less than that of the passaged strains. It is concluded that virulence increase of pigeon PMV-1 strains during passages in chickens is due to selection of the most virulent subpopulation.

Keywords: Pigeon PMV-1, virulence increase, selection, heterogeneity

Newcastle disease virus (NDV) strains are composed of heterogeneous virus populations with respect to plaque size (Granoff, 1964), plaque type (Daniel and Hanson, 1968), heat stability (Granoff, 1964; Spalatin and Hanson, 1976), and virulence (Daniel and Hanson, 1968; Prokopovitsch and Lomniczi, 1973; Hanson and Spalatin, 1981; McMillan and Hanson, 1986). The fluctuation in the virulence of a natural NDV isolate during passage in eggs (Hanson and Spalatin, 1981) and the reduction of its virulence during passage through chickens (Brough and Beard, 1984) have been explained by heterogeneity. Alexander and Parsons (1984) were the first to report an increase of virulence during passage of pigeon paramyxovirus-1 (PMV-1) in chickens; they, however, did not investigate the mechanism underlying this phenomenon.

An increase in the virulence of pigeon PMV-1 strains has great epizootiological importance and it has also been observed by us with some Hungarian isolates of NDV (Kissi, 1988). In the present work the mechanism of the virulence increase observed in chickens was investigated. It has been revealed that some PMV-1 strains are heterogeneous in virulence and that the virulence increase occurring upon passage in chickens is caused by selection of the most virulent virus clones.

Materials and methods

Virus propagation, cell culture work and animal experiments were performed as described in the accompanying paper (Kissi, 1988).

Results

Identification of the most virulent plaques in the virus population of strain 58

It has been assumed that upon passage in chickens the most virulent virions of a heterogeneous population will overgrow the less virulent ones and, as a result of selection, after a few passages the former will constitute the majority of the population. Whether this is true or not can easily be ascertained if we select plaques of a strain showing increase in virulence, grow them in embryonated eggs, and determine their virulence in chickens. As, however, this would require at least 2 to 3 growing chickens per sample, and it is questionable whether the proportion of highly virulent plaques would reach even 1 per cent, the plaques of a single strain would have to be tested in several hundred growing chickens, a procedure that would obviously be far too expensive.

Therefore, an economical method was employed for identifying the most virulent plaques within a given virus population. Briefly: 50 or 100 plaques were picked out from several strains showing an increase in virulence (strains 55, 58 and 234). Each plaque was grown up in an embryonated egg, and each allantoic fluid was marked separately (e. g. 55 pl, p2, p3, etc.). Subsequently 0.1 ml volumes were taken from each plaque isolate and pooled in groups of ten (1 to 10, 11 to 20, etc.). Each pool of ten plaques was inoculated into three chickens. If a group contained at least two positive (ill or dying) birds, all 10 plaques of the given pool were examined separately. Plaques of the highest virulence were identified by inoculating three chickens each, and IVPI tests were done on each plaque in six birds.

The procedure is demonstrated in detail on strain 58 (Table I). It can be seen that initially the IVPI of strain 58 had been O. The virulence of strain 58 plaque mixture p31–p40 already exceeded that of the initial strain (2 positive out of 3 birds tested). In plaque mixture p31–p40 p33 and p39 had an IVPI of 0.8 and 1.1, respectively, indicating that strain 58, an isolate with an IVPI = 0, contained a plaque of at least as high a virulence as IVPI = 1.1. Although this is lower than 1.7 (the IVPI measured for strain 58 after five passages in chickens), the chances of finding the most virulent plaque are very poor (let alone the excessive number of experimental birds to be used). However, this is not necessary for proving the above hypothesis.

Table I

Identification of the most virulent virus in pigeon PMV-1 population designated 58

A) Pathogenicity of virus mixtures

Virus plaque mixtures	Chickens		
	number inoculated	number paralysed	number dead
p1–p10	3	0	1
p11–p20	3	0	0
p21–p30	3	0	1
p31–p40	3	1	1
p41–p50	3	0	0

B) Pathogenicity of virus plaques

Virus plaques	Chickens		
	number inoculated	number paralysed	number dead
p31	3	0	1
p32	3	1	1
p33	3	1	1
p34	3	0	0
p35	3	0	1
p36	3	0	0
p37	3	0	1
p38	3	0	1
p39	3	1	1
p40	3	0	1

C) Virulence of individual plaques

Plaque	Chickens			Virulence (IVPI)
	number inoculated	number paralysed	number dead	
p33	6	0	3	0.80
p39	6	2	2	1.10

Heterogeneity in virulence of PMV-1 strains 55 and 234 before and after passage in chickens

Table II shows the heterogeneity in virulence of strain 55, while Table III summarizes the results obtained with the three strains.

In can be seen that by analysing 100 plaques each strain was found to contain plaques of much higher virulence than the initial virulence level, even

Table II

Virulence distribution of 100 plaques isolated from strain 55

Mixture of plaques	IVPI	Individual plaques	IVPI
1–10	0.0	p11	0.0
11–20	1.0	p12	0.0
21–30	0.0	p13	*0.3*
31–40	0.0	p14	0.2
41–50	0.2	p15	0.2
51–60	0.2	p16	0.0
61–70	0.4	p17	0.0
71–80	0.4	p18	0.0
81–90	1.0	p19	*0.9*
91–100	0.0	p20	0.0

Table III

Plaques of the highest virulence of different pigeon PMV-1 strains

Virus strain or plaque	Virulence (IVPI)	
	Initial inoculum	5th passage
55 original	0.0	1.3
p13	0.3	
p19	0.9	
58 original	0.0	1.7
p33	0.8	
p39	1.1	
234 original	0.6	1.8
p24	1.1	
p26	1.2	

if their virulence failed to reach that found after passage in chickens. When plaques were picked out from such strains of increased virulence, the individual virulence of these plaques was always high and the strain in question could be considered rather homogeneous (data not shown).

Discussion

From the epizootiological point of view the most interesting property of pigeon PMV-1 strains is that during passage through chickens their virulence increases (Alexander and Parsons, 1984; Bijnens et al., 1985; Kissi, 1988).

The results of the above authors suggest that in respect to heterogeneity pigeon PMV-1 strains fall into two categories: (a) Some strains are probably homogeneous in virulence and do not contain populations of higher virulence than the original strain. Therefore, during passage in chickens the virulence of such strains will not change. (b) Strains which undergo a substantial increase in virulence during passage in chickens. These strains are heterogeneous in virulence and during passage in chickens the most virulent subclones of the population are rapidly selected for. This is not inconsistent with the fact that with the three strains examined by us the analysis of 100 plaques was not sufficient to find plaques of the highest virulence (i.e. those of post-passage virulence level) in the strains before passage in chickens. This must have been due to the fact that in none of the strains did the proportion of the most virulent plaques reach 1%. At the same time, the most virulent plaques of the original population were virulent enough (though still within the mesogenic category) to prove that these strains were heterogeneous in virulence.

It should also be noted that heterogeneity in virulence *per se* does not explain the mechanism of virulence increase during passage in chickens and does not mean that enrichment and selection of the most virulent subclones are obligatory steps. On the contrary, there have been reports on the decrease in virulence of NDV strains passaged in chickens. E. g. Brough and Beard (1984) isolated a viscerotropic velogenic NDV strain from exotic birds. This strain caused only mild disease signs in chickens and, interestingly, its virulence was not increased even after passage in chickens.

Investigating the heterogeneity of an NDV strain, Hanson and Spalatin (1981) established that the strain was composed of mesogenic and velogenic subpopulations. After separation of these subpopulations and their combined inoculation in a given ratio it was found that clones of lower virulence inhibited the virulence of the more virulent subpopulations.

Hence, pigeon PMV-1 strains differ from the above NDV strains in their biological property mentioned above. Namely, several pigeon PMV-1 strains have a latent virulence for chickens, which manifests itself after passage in this species. It has been established that heterogeneity in virulence and rapid selection of the most virulent subpopulation in chickens are the factors underlying the increase in virulence. Such a selection may also occur, though much less rapidly, during passage in embryonated eggs (Kissi, 1988). Even if the less virulent subpopulations are predominant within a pigeon PMV-1 strain, the most virulent subpopulations will overgrow them, contrary to what was found for NDV strains studied by others. It is to be noted that a mesogenic NDV strain, *Roakin*, exhibits a similar phenomenon (data not shown).

As opposed to what has been reported on the changes of mixed NDV populations, pigeon PMV-1 subpopulations of higher virulence for chicken will rapidly overgrow the less pathogenic ones in a susceptible host since the lat-

ter do not exert any inhibitory effect (e.g. by interference). The fact that pigeon PMV-1 strains carry subpopulations whose chicken pathogenicity can be activated rapidly has practical significance as a potential threat to chicken flocks. However, this phenomenon may also indicate that the emergence of heterogeneity of PMV-1 strains is facilitated in the pigeon. The latter possibility has been tested experimentally: two strains failing to undergo virulence increase during passage in chickens did so upon passage in pigeons. After five passages this increase was not too high (the IVPI of one of the strains rose to 1.0). Still, since the virulence increase occurred for a strain that failed to yield virulent subpopulations by passage through chickens, it can be assumed that this recent heterogeneity has emerged during passage in pigeons (Kissi,1988).

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BOOK REVIEWS

A. SCHEINERT and A. TRAUTMANN: *Textbook of Veterinary Physiology*. 7th Edition. Edited by G. Wittke. Paul Parey, Berlin und Hamburg, 1987. ISBN 3-489-66216-4. 712 pages, index, with 418 graphs, 2 coloured tables and 116 tables.

The textbook was first published in 1939 by Scheunert, Trautmann and Krzywanek. The recent 7th edition has been edited by Prof. Wittke and is also fully reconsidered as a real textbook of veterinary physiology.

The subject is detailed in the following 15 chapters: I. Introduction. II. Basis of life-processes. III. Metabolism. IV. Thermal regulation. V. Blood and lymph. VI. Blood circulation. VII. Respiration. VIII. Water and electrolyte regulation, renal physiology. IX. Endocrines. X. Physiology of reproduction. XI. Physiology of milk secretion. XII. Egg formation. XIII. Neurophysiology and sensory functions. XIV. Movement. XV. Physiology of behaviour.

References are added to each chapter and a detailed index will help the readers find the requested data. The concept of this book is quite clear. The logical "build-up" of the text makes easy for the reader to understand the important facts of physiology. New parts are added as e.g. Physiology of behaviour. The book was fully revised, especially new data on neurophysiology, endocrinology, milk secretion, egg formation and metabolism are detailed impressively. Tables, figures and other illustrations are presented in an easily understandable manner.

Typographically the two-column pages and the page setting of the text, often used by Springer Verlag, makes reading and learning very easy.

The book is intended for the education of veterinary students. It offers an excellent description of the main topics of physiology. This book is therefore very much recommended for German-speaking students as well as for veterinarians, animal production experts, and especially for use in postgraduate training.

The book was written by 16 authors (E. Bamberg, K. Bronsch, H. Eder, M. Fromm, J. Gropp, K. Hierholzer, H. Hörnicke, G. Hofecker, K. Männer, E. Pfeffer, E. Scharrer, H. Spörri, A. Wels, G. Wittke, K. Zerobin, and H. Zucker). Despite the relative large number of authors the language of the text is very homogeneous. For this fact the Editor, Prof. Dr. G. Wittke deserves praise.

G. PETHES

ROY MACK: *Dictionary for Veterinary Science and Biosciences/Wörterbuch für Veterinärmedizin und Biowissenschaften*. German-English/English-German/With trilingual appendix: Latin terms. By R. Mack, Weybridge/England. 1987. 324 pages. Soft cover DM 49.80. ISBN 3-489-50516-6.

This dictionary has been compiled as a result of many years experience of translating texts in the biological sciences, particularly veterinary medicine. The aim is to supplement the general dictionaries with technical terms in the fields of anatomy, microbiology, physiology, parasitology, pathology, pharmacology, toxicology and zootechnics, with special reference to domestic animals and their diseases. The commoner wild animals and those present in zoos are also included. As a specialized vocabulary the work is addressed to students, practicians and scientists in veterinary medicine and in the biological sciences; it is indispensible to professional translators, libraries and authorities.

PAYNE, J. M. and PAYNE, S.: *The Metabolic Profile Test.* Oxford University Press, New York. 1987. 179 pages.

By surveying relevant papers published since 1970, the authors give an account of the new biological concept in which the use of profile tests occupies a central place. This new concept stems from experience that subclinical metabolic disorders disturb the production of domestic animals more than do clinically apparent diseases. In the absence of clinical symptoms these disorders are demonstrable only by laboratory chemical tests elaborated in recent years. The authors evaluate the results of these investigations, summarize recent years' findings, and predict possible future applications of this new biological concept.

Profile tests were first developed in the field of cattle production where the results are differentiated according to aspects of milk and beef production. Subsequently parameters best reflecting the intensity of production were established for other species of animals, too.

The book is divided into 18 chapters devoted to the critical evaluation of parameters used in the profile tests. These are as follow.

The background of profile tests (critical evaluation of the profile tests). *Indicators of energy status* (glucose, non-esterified fatty acids, volatile fatty acids). *Indicators of protein status* (urea, albumin, globulin). *Indicators of calcium and phosphorus. Indicators of magnesium. Indicators of the electrolytes* (Na, K). *Haemoglobin and packed cell volume as indicators. Indicators of trace elements* (Cu, Se, I, Co, Zn, Mn). *Indicators of blood enzymes* (GOT, GPT, LDH, CPK). *The profile test as a monitor of exercise. Profile tests to monitor growth. Prediction and diagnosis of disease. Profile tests for infertility. Detection of stress. The profile test used in surveys. Profile tests in the detection of congenital disorders. Profile tests in various species. The future of profile tests.*

The book provides abundant information for basic and applied research of the production and production diseases of domestic animals.

L. PROHÁSZKA

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