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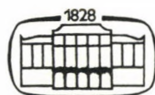

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1983

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FOREWORD

With the present volume, the Reader receives our Journal in a modernized form. The Editorial Board has attempted to evolve the most commonly accepted forms of publication.

For this purpose, the name of the journal has been shortened. Instead of the formerly used Acta Veterinaria Academiae Scientiarum Hungaricae, in the future the journal will be published under the name *Acta Veterinaria Hungarica* (Acta Vet. Hung.), thereby making easier its citation.

Since in the future papers will be published exclusively in English, the subheadings of Contents will also be indicated in English and not in Latin.

The first page of the papers will contain the title of the paper and the name(s) of the author(s), followed by the English abstract and the keywords commonly used in the international scientific literature.

Instructions to authors (guidelines on manuscript form and style) will regularly appear on the inside back cover. In order to raise the standard of the papers, they will be subject to stricter judgement by referees in the future.

As regards the contents of the papers, we should like to give a picture of veterinary research activities associated with intensive animal breeding. During the last decade, Hungary has achieved great progress in this field. This is indicated also by the fact that, as regards per capita meat production, Hungary belongs to the vanguard of the world. The continuation of research activities in this direction deserves attention.

In our age, science keeps becoming more and more specialized, raising difficulties for those who want to get general information. Therefore, we endeavour to regularly publish in our Journal also high-standard reviews and surveys written by the best specialists.

The Journal publishes mainly Hungarian research results, but papers of foreign research workers which fit into the scope of the Journal are also welcome. Naturally, only original papers not published, submitted or accepted for publication elsewhere can be taken into consideration.

Papers published in Acta Veterinaria Hungarica have a wide publicity since the Journal is covered by all international abstracting journals and centres. This publicity is facilitated by the fact that one hundred reprints of each paper are supplied free of charge.

We hope that Acta Veterinaria Hungarica, which will appear in an up-to-date form and with contents of higher standards, will find our readers' approval.

Budapest, May 1983

János Mészáros
Editor-in-Chief

Acta Veterinaria Hungarica 31 (1—3), pp. 5—15 (1983)

ISOLATION OF PORCINE PARVOVIRUS (PPV) FROM SWINE HERDS AFFECTED BY REPRODUCTIVE FAILURE, AND SEROLOGIC EVIDENCE OF INFECTION IN HUNGARIAN LARGE SWINE HERDS

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(Received March 4, 1983)

The SMEDI syndrome was diagnosed in two large-scale farms and in a private stock. The clinical picture was characterized by stillbirth, embryonic and fetal death, mummification and infertility, but no abortion or premature delivery occurred. Particularly litters of young, primiparous sows were affected. while those of repeatedly bred sows remained symptomless. In two large-scale farms, the rate of stillborn and mummified fetuses was 8.8 and 5.8%, respectively, of the piglets born alive during the period of examination. In 9 and 11 cases out of 14 mummified fetuses, PPV antigen was demonstrated by the haemagglutination test and by direct immunofluorescence of cryostat sections of fetal tissues, respectively. PPV was isolated from the organs of 9 fetuses in swine kidney and thyroid cell cultures. The isolates were identified by haemagglutination-inhibition (HI) and fluorescent antibody tests and by electron microscopic examination. HI antibody to PPV was found in the body fluids of all the stillborn piglets died in late pregnancy and its titre reached or surpassed 1 : 400 in all blood sera collected from dams of affected litters. The role of other agents known to be associated with reproductive disorders in swine was ruled out by a series of laboratory examinations.

Serological screening carried out in 42 large-scale farms between 1976 and 1981 indicated a wide distribution of PPV infection in the Hungarian swine population. The average percentage of seroconverted swine was 83.5%. Both the incidence and the titre of PPV HI antibodies were significantly higher in herds with reproductive losses than in the apparently healthy ones: all the sera tested from 5 affected herds had HI antibody to PPV, the great majority of them (95.2%) in a titre of 1 : 1600 or higher. It is concluded that PPV plays an important role in the aetiology of reproductive disorders of swine in Hungary.

Keywords. Porcine parvovirus infection, swine, reproductive disorders, virus isolation, serology.

Normal development of swine fetuses can be disturbed by virus infections. Some of the viruses, including hog cholera, foot-and-mouth disease and Aujeszky's disease viruses, cause obvious clinical symptoms and in pregnant sows the acute clinical disease may be accompanied by abortion and stillbirth. In contrast, a number of viruses are known to produce embryonic and fetal death but do not cause abortion or premature delivery. These viruses appear to have generally little or no pathological effect on swine, including pregnant sows, thus the reproductive disorders may escape the attention of swine breeders. The term SMEDI syndrome was originally used by Dunne et al. (1965) to

designate *s*tillbirth, *m*ummification, *e*mbryonic *d*eath and *i*nfertility caused by porcine enteroviruses. Since then it has been shown that various viruses are associated with SMEDI syndrome, including attenuated hog cholera virus (Young et al., 1955), Japanese encephalitis virus (Matumoto et al., 1949; Shimizu and Kawakami, 1949), Sendai virus (Shimizu et al., 1954) and foot--and-mouth disease virus (Mickowitz, 1965), and that the porcine parvovirus (PPV) has a major role in reproductive problems of swine. Cartwright and Huck (1967) isolated 111 virus strains from swine herds with reproductive disorders. Ninety-six of the isolates proved to be PPV. Isolation of PPV from swine herds with reproductive problems has been reported from countries of several continents, namely, the United States (Mengeling, 1972), Japan (Morimoto et al., 1972), Australia (Coackley and Smith, 1972), the Netherlands (Rondhuis and Straver, 1972), South Africa (Pini, 1975) and New Zealand (Horner and Hunter, 1977).

In Hungary, serological surveys have suggested a wide distribution of PPV and its remarkable role in the aetiology of reproductive problems observed in swine herds free of brucellosis and leptospirosis (Kudron and Mocsári, 1979). Attempts to isolate the virus, however, have failed until recently.

This paper is the first report on the isolation of PPV in Hungary, from swine herds affected by reproductive failure. Results of serological screening on the incidence of PPV infection in large swine herds are also presented.

Materials and methods

Specimens. The specimens were collected in two large-scale farms with 265 and 161 breeding sows, respectively, and in a private backyard farm of 5 sows. A total of 31 fetuses were examined: 14 mummified and 17 stillborn ones. Examination of tissues by direct immunofluorescence and virus isolation was attempted from the mummified fetuses. Body fluids and tissue homogenates of fetuses died after 70 days of gestation were tested for PPV haemagglutination inhibiting antibody. For the serological screening, 4080 blood samples were collected in 11 of the 19 counties of Hungary, in 42 large swine herds, during a 6-year period (1976–1981).

Haemagglutination (HA) test. Homogenates of lungs, liver and spleen of each fetus were tested for HA activity as described by Joo et al. (1976), using guinea-pig erythrocytes. Homogenates showing HA activity were treated with 20% (v/v) chloroform and the HA test was repeated. HA activity was inhibited by a reference anti-PPV serum prepared in rabbits with the NADL-2 reference strain of PPV (Mengeling and Cutlip, 1975).

Immunofluorescence (IF). The direct method was applied. Small pieces of lungs and spleen were embedded in Cryoform™ (DAMON, IEC Division,

Needham, USA). Cryostat sections were fixed in a mixture of 1 part of methanol and 3 parts of acetone for 10 min at room temperature. The anti-PPV FITC conjugate was prepared from the IgG fraction of the reference anti-PPV serum according to the method of Clark and Shepard (1963). Specificity of the fluorescent antibody reaction was confirmed in each case by also reacting cryostat sections with the mixture of FITC conjugate and anti-PPV serum.

Virus isolation attempts. Secondary cultures of porcine kidney and thyroid cells were used. The isolation procedure reported by Mengeling et al. (1979) was slightly modified. Namely, the secondary cultures were prepared 24 h after the inoculum had been added to the nutrient medium of primary monolayer cultures. The secondary cultures were incubated at 37 °C for 7 days. The cell-associated virus was released by a few rapid freezing-and-thawing cycles, the cell debris were removed by centrifugation and the supernatant fluid was tested for HA activity. Simultaneously, coverslip cultures prepared in Leighton tubes were inoculated and subsequently examined by IF 12 to 24, 48 and 72 h post inoculation. Cytopathic effect of the isolates was studied in haematoxylin-eosin (HE) stained coverslip cultures.

Electron microscopic (EM) examination. Homogenates of virus-infected secondary porcine kidney cells were centrifuged by $100{,}000 \times g$ for two hours at 4 °C. The pellet was resuspended in one drop of distilled water, mounted on formwar-coated grids and stained with 2% phosphotungstic acid. The grids were examined in a Philips 201 CS electron microscope operating at an acceleration potential of 60 kV.

PPV antigen. The NADL-2 reference strain of PPV was used. The virus was propagated in secondary porcine kidney cells as described above. The antigen preparation was similar to the method of Joo et al. (1975). It was stored in small aliquots at −20 °C and titrated immediately before use.

Haemagglutination-inhibition (HI) test. The standard method of Joo et al. (1976) was slightly modified. Heat-inactivated test sera were diluted 1 : 25 and absorbed only with a 10% suspension of guinea-pig erythrocytes. Twofold dilutions were prepared in WHO HA plates in volumes of 0.4 ml (1 : 50 to 1 : 51,200). As diluent, buffered isotonic saline (Linsert et al., 1970) was used throughout. The test was performed with 4 HA units of PPV antigen. The mixtures of virus–serum dilutions were incubated at 37 °C for 60 min. After having added the guinea-pig erythrocytes, the plates were incubated at room temperature for another 60 min and the test was read. According to previous experience (Joo and Johnson, 1976; Vannier et al., 1976), only titres of 1 : 400 or higher were considered positive. As HI antibody titre, the highest serum dilution was considered that entirely prevented the HA.

Results

Embryonic and fetal death and reproductive problems dominated in each farm. Abortion and premature delivery did not occur, whereas post-term delivery was a frequent finding. In certain litters, besides a few live-born piglets, fetuses died at different times of gestation and mummified fetuses were also delivered. The size of mummified fetuses was between 8 cm and the size of a mature fetus. Their colour varied from dark brown to the colour of a healthy fetus. The body mass of live-born piglets was generally subnormal. Milk production of sows was undisturbed, however, the suckling activity of the piglets was reduced. Postnatal losses were also unusually high. The sows, and generally the swine stocks, however, showed clinical symptoms neither before the appearance of reproductive losses, nor in the course of their observation. The percentage of sows that had failed to conceive was high (approx. 45%) in both affected large swine stocks. Principally, young sows in the first or second gestation were affected, the older ones remained symptomless. Some data on the incidence of the condition are shown in Table I.

Table I

Data on the course of disease in three swine stocks with reproductive failure

Stock	Number of sows in the farm	Number of affected sows	Farrowing data			
			Number of mummified fetuses	Number of stillborn pigs	Number of live-born pigs	Number of fetuses examined
A	265	10	52	29	17	7/11+
B	161	13	40	32	46	4/3
C	5	2	13	18	—	3/3
Total	431	25	105	79	63	14/17

+ = Number of mummified fetuses/Number of fetuses died at different times of gestation

The tissue homogenates of 9 out of 14 mummified fetuses showed *HA activity*. The HA titres of the organs varied from 1 : 16 to 1 : 512. The HA activity was not affected by chloroform treatment, and it could specifically be inhibited with the reference anti-PPV serum.

PPV antigen was demonstrated by *IF* in the organs of 11 fetuses (Fig. 1). The specific reaction was blocked with anti-PPV serum in all cases.

PPV was isolated from the organs of the 9 fetuses showing HA activity. The *cytopathic effect* (CPE) of the isolates was not characteristic and it became evident both in porcine kidney and thyroid cell cultures only after two or three blind passages. The most consistent alterations of virus-infected cells such as shrinkage and necrosis were similar to the degenerative changes that

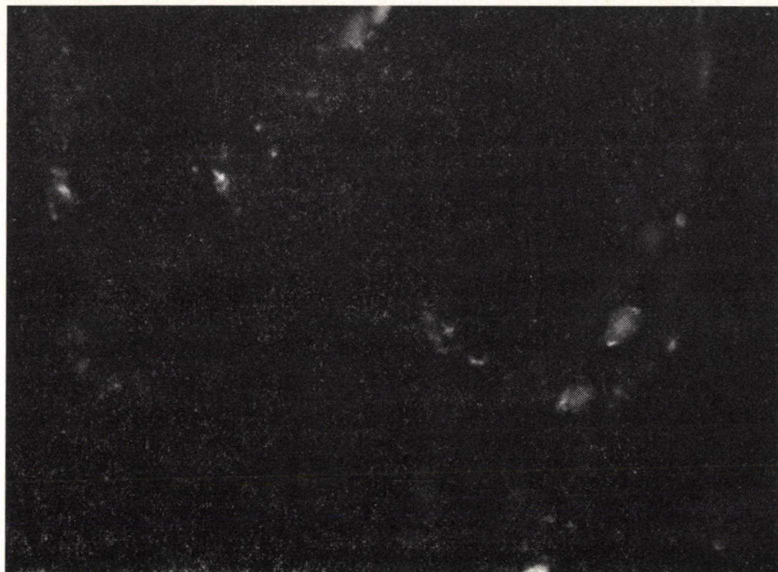

Fig. 1. Cryostat section of the lungs of a mummified fetus. Fluorescing PPV antigen in the alveolar cells. ×640

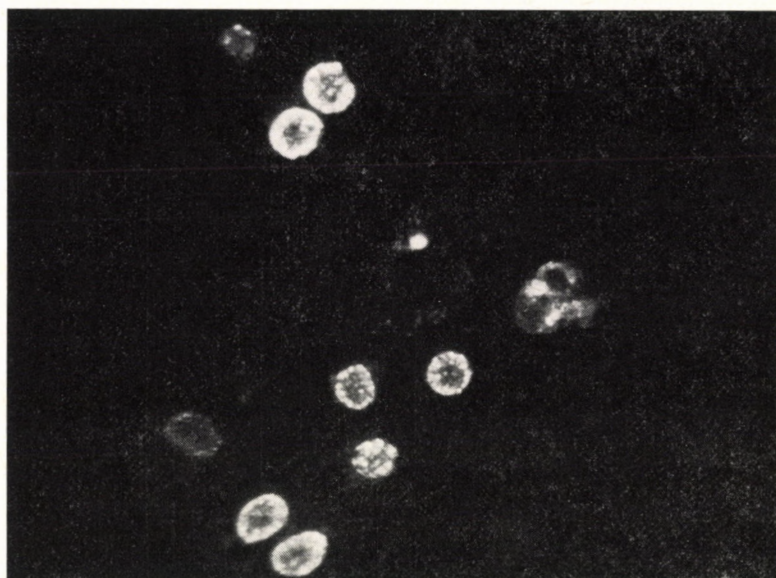

Fig. 2. IF staining of a porcine kidney cell culture 18 h after infection with one of the PPV isolates. Viral antigen is localized almost exclusively in the nuclei of infected cells. ×640

occurred in uninfected aged cell cultures. In HE-stained preparations, however, characteristic Cowdry type-A intranuclear inclusions were found.

All the isolates agglutinated guinea-pig erythrocytes and the anti-PPV serum inhibited the HA activity. Six and three isolates showed HA activity in the first and second passage, respectively.

By *IF examination of the coverslip cultures*, PPV could be identified in all cases in the first passage. In cultures fixed 16 to 24 h post-inoculation, definite condensation of viral antigen in the nuclei was the most characteristic finding (Fig. 2); in later stages of infection, however, PPV antigen was equally detected in the nucleus and the cytoplasm of the infected cells.

The *electron microscopic examination* revealed naked icosahedral virus particles with an average diameter of 20 to 22 nm (Fig. 3), matching parvovirus morphology and size.

Fetal PPV HI antibody was found in the body fluids and tissue homogenates of all the examined 17 stillborn piglets died after the 70th day of gestation. Their titres varied between 1 : 16 and 1 : 128, mostly 1 : 64 and 1 : 128 (in 13 fetuses, 76.5%).

HI titres of 74 blood sera collected in the three farms from dams of the affected litters are shown in Fig. 4. All the sera had HI antibody in a titre of at least 1 : 400, the majority of them (67.6%) in a titre of 1 : 1600 or higher.

In the three farms, the role of another known agent associated with reproductive failure in swine (e.g. brucellae, leptospires and Aujeszky's disease virus) was ruled out by bacteriological, virological and histological examina-

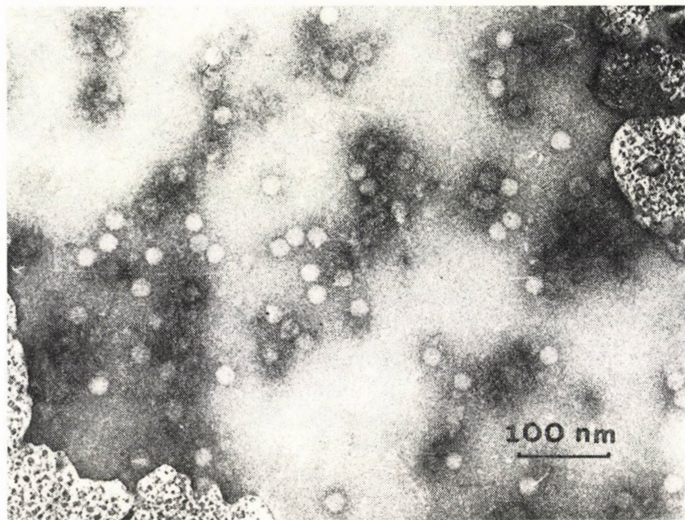

Fig. 3. Naked virus particles with an average diameter of 20 to 22 nm in the homogenate of a swine kidney cell culture infected with one of the PPV isolates. Negatively stained preparation

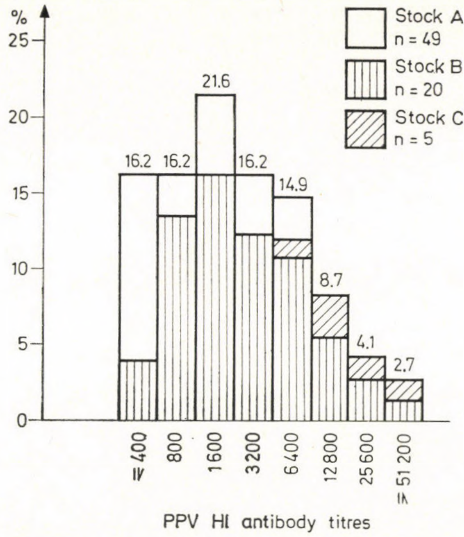

Fig. 4. Percentage distribution of PPV HI antibody titres in groups of sows with reproductive failure

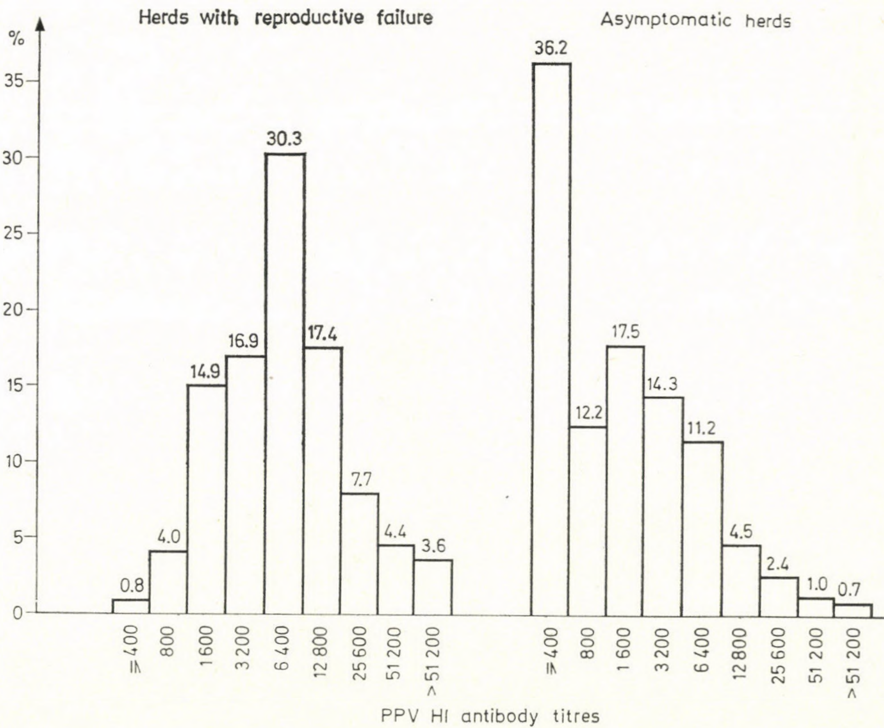

Fig. 5. Percentage distribution of PPV HI antibody titres in 1067 blood sera originating from 5 herds affected by reproductive disorders and from 13 asymptomatic herds

Table II

Incidence and yearly distribution of PPV HI antibody in 42 large swine herds between 1976 and 1981

Years	Blood samples	
	Examined (n)	Positive (%)
1976	413	65.6
1977	537	86.2
1978	676	84.9
1979	376	82.7
1980	818	84.3
1981	1260	87.1
Total	4080	83.5

tions and also by serological examination of blood sera collected from the dams of affected litters.

Results of the *serological screening for the incidence and yearly distribution of PPV HI antibody* are shown in Table II. All the herds examined proved to be infected by PPV. Out of the 4080 blood sera tested, 3406 (83.5%) had a PPV HI antibody titre of 1 : 400 or higher.

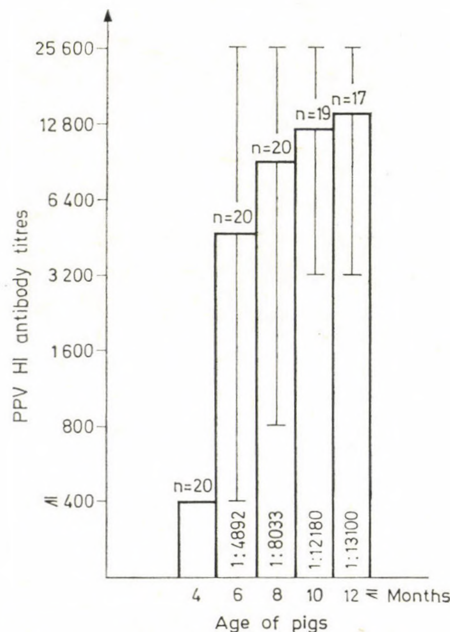

Fig. 6. **Distribution of PPV HI antibody titres in pigs of different ages in a large swine stock endemically infected by PPV**

HI titres of sera from 5 herds affected by reproductive failure were compared to those originating from 13 herds without a history of reproductive losses (Fig. 5). In the herds with reproductive losses, HI titres lower than 1 : 400 were found in none of the 248 sera tested and only 2 (0.8%) had a titre of 1 : 400. On the contrary, in 819 sera collected in apparently symptomless herds 251 (30.6%) had PPV titres lower than 1 : 400 and 45 (5.5%) a titre of 1 : 400.

Distribution of PPV HI antibody in 100 pigs of different ages and their mean geometric titres are presented in Fig. 6. The sera were collected in an endemically infected farm with 2000 productive sows.

Discussion

During the past few years, reproductive failures were observed in large swine herds free of brucellosis and leptospirosis. The reproductive problems were accompanied by embryonic and fetal death, mummification and infertility, however, isolation, or IF demonstration, of PPV failed.

Nowadays, there is a great demand for pregnant gilts raised in farms free of brucellosis, leptospirosis and Aujeszky's disease virus infection. Besides, swine breeding in backyard farms shows a continuous upward trend. To meet the demands and make the production more profitable, more gilts were selected from the fattening units for breeding, and their first mating was done at an age earlier than usual, at 6 to 7 months of age. At this age, a considerable part (2 to 47%) of gilts are still susceptible to PPV infection (Joo and Johnson, 1976). Thus, embryonic and fetal death and reproductive losses are more frequently observed in these animals than in gilts bred later, after 7 to 8 months of age, by the time active immunity has already developed in almost all the pigs in endemically infected farms.

In the two large-scale farms where PPV was isolated, 3.8 and 8.1% of the sows showed reproductive disorders and the rate of stillborn and mummified fetuses was 8.8 and 5.8% of the piglets born alive during the period of examination, respectively. The data are comparable to those reported from abroad. In Australia, e.g., Gillick (1977) found a 9.5% incidence on the average, in 3000 sows of four large-scale farms endemically infected by PPV. The rate of stillborn and mummified fetuses varied in each farm from time to time between 0.2 and 20%.

In the backyard farm, PPV infection was introduced by pregnant gilts purchased from a large-scale farm. Out of three own-bred sows, two showed reproductive disorders, the third and the two purchased gilts remained symptomless, their litters were normal.

Results of serological screening have indicated a wide distribution of PPV in Hungarian large swine herds. The average percentage of seroconverted

swine (83.5%) corresponds to that reported for the swine population of other countries (Joo and Johnson, 1976). Yearly distribution of seroconverted animals was similar throughout the examination period except for the first year (1976), when testing of groups of animals was less selective, and mainly young animals were tested.

In herds with reproductive problems, both the percentage of seroconverted animals and the PPV HI antibody titres were significantly higher ($P < 0.05$) than in the apparently healthy ones. These findings make it highly possible that the observed reproductive disorders have been associated with PPV even in those farms in which the isolation or IF demonstration of PPV failed.

Results of the serological study carried out in pigs of different ages raised in an endemically infected large swine herd have shown that most of the pigs become infected after 4 months of age and they are still susceptible up to, or past, the stage of sexual maturity (6 to 7 months of age).

In addition to clinical and epizootiological studies, experience was gained as to the virological diagnosis of PPV-induced reproductive failure. Results of direct HA test with fetal tissue homogenates corresponded to the results of virus isolation attempts and, except two cases, also to those of IF test. The test has been found suitable for quick demonstration of PPV antigen. Demonstration of PPV HI antibody in body fluids and tissue homogenates of immunocompetent fetuses similarly supports the causative role of PPV in reproductive disorders.

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EXPERIMENTAL INFECTION OF CHICKEN EMBRYOS AND DAY-OLD CHICKS WITH A LENTOGENIC STRAIN OF NEWCASTLE DISEASE VIRUS

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Fifty SPF chicken embryos were infected with the B_1 strain of Newcastle disease virus (NDV) on the 6th day (group A), another fifty on the 13th day of incubation (group B), and 25 chicks of the same controlled stock on the 2nd day after hatching (group C). Of each group, the appropriate organs of three embryos or chicks were examined by morphological methods every second day after infection. Earlier results of the authors, obtained in studies on normal embryos and chicks, were used for comparison. The presence of the virus in embryos and chicks was demonstrated by the haemagglutination (HA) and immunofluorescence (IF) tests and virus isolation.

Local infiltration with lymphoid cells in some organs, lymphoblast proliferation in the developing lymphoid follicles, and tissue plasmacytosis indicative of humoral immune response were shown to occur only around the time of hatching and after hatching, irrespective of the time of infection. Prior to this time, between the 6th and 10th day of incubation (group A) only signs of circulatory disturbance were observed. Between the 11th and 19th day of incubation (groups A and B), enhanced granulocytopoiesis was seen in the bursa of Fabricius and spleen, accompanied by a pseudoeosinophil granulocytic interstitial pneumonia and disturbances in the development of lymphoid organs and tissues (delayed and uneven lymphocyte colonization in the follicles of the bursa of Fabricius, degeneration and necrosis of lymphocytes).

In group A, a part of the embryos died between the 3rd and 5th day, while in group B on the 6th day, after infection. In group A, the development of embryos was markedly retarded, and this was seen also at hatching. The highest antibody titres were demonstrated in group C.

Keywords. Experimental infection, chicken embryos, pathological studies.

In previous studies (Glávits et al., 1982) we demonstrated granulocytopoiesis in the interstices of the bursa of Fabricius of chicken embryos from the 10th day of incubation on. Simultaneously with the colonization of lymphocytes in the developing lymphoid follicles, granulocytopoiesis gradually falls into the background, and ceases completely around hatching. In the present work, we studied the effect of a viral infection on the myeloid and lymphatic system of chicken embryos and day-old chicks in different stages of incubation and after hatching. In addition to this, the types of cellular reactions, the development of immune responsiveness and the characteristics of pathological lesions were studied in chicken embryos, with regard to the developmental status of these organ systems.

Materials and methods

A total of 120 dwarf Leghorn chicken embryos and 50 chicks derived from controlled (SPF) stocks were used in the experiments. The eggs were incubated in Ragus 60 laboratory incubators.

Infection. Fifty embryos each were inoculated into the allantoic sac with 100 EID (embryo infective dose) of the Newcastle disease virus strain B_1 on the 6th (group A) or 13th (group B) day of incubation. The inoculation volume was 0.1 ml. Twenty-five one-day-old chicks (group C) were infected through the aerogenic route with material of the same dilution. Every second day after infection, two embryos each of groups A and B were subjected to gross pathological and histological examination, while one embryo of each group was used for electron-microscopic and virological examinations. The embryos found dead at candling performed twice daily were examined histologically and used for bacteriological, mycological and virological examinations.

From hatched chicks belonging to group A and B, and also from those of group C, samples for the above examinations were taken on the 1st, 2nd, 5th and 10th days after hatching, as described earlier (Glávits et al., 1982). In the evaluation of the experiment, in addition to uninfected control embryos and chicks, the results of our previous work were used for comparison (Glávits et al., 1982).

Histological and electron-microscopic examinations. These, too, were performed as described previously (Glávits et al., 1982), with the exception that also lung samples were taken for electron microscopy.

Virological examinations. The virus content of the allantoic fluid of infected embryos was determined by the haemagglutination (HA) test. Sections prepared from the tracheal mucosa of embryos in the late phase of incubation and from that of the spray-infected day-old chicks were examined by direct immunofluorescence (IF). Virus isolation was attempted from embryonated eggs. Specific serum antibody titres of 4 survived chicks of both groups A and B, and of 15 chicks of group C, were determined by the haemagglutination-inhibition (HI) test at 10 days of age.

Results

The presence of the virus strain used for infection in the allantoic fluid of killed or died embryos of both groups A and B was demonstrated by the HA test up to the 18th day of incubation.

Subsequently, the virus was detected by the IF method or virus isolation on post-hatching days 1 and 2 in group A, and even on day 5 in group B. From chicks belonging to group C, virus isolation was successful from the

48th h up to the 5th day postinfection, but in organ samples taken on the 10th day postinfection the presence of virus could be demonstrated neither by virus isolation nor by any other method.

In organs of embryos belonging to *group A*, and killed or died between the 6th and 10th day of incubation, minor haemorrhages and, in some places, oedema were observed, as compared with the controls. Between the 12th and 16th day of incubation, the lungs of all infected embryos were found to contain more or less heterophil granulocytes in the interstices, primarily around blood vessels (Figs 1 and 2). Similar changes were occasionally seen also in other organs (liver, spleen, kidney, glandular stomach wall, intestinal wall, myocardium, meninges, etc.) of some embryos. In the embryonal mesenchyma of the developing bursa of Fabricius, still free of lymphoid follicles, and in the reticular tissue of the spleen, leucocyte forms representing different stages of granulocytopoiesis (myeloblasts, promyelocytes, metamyelocytes) were present in larger numbers and density than in the controls. Between the 18th and 20th day of incubation, disturbance of the development of lymphoid tissue was observed in the bursa of Fabricius. The rate of lymphocyte colonization in the developing follicles was uneven and differed from that of the control (Fig. 4). Small follicles consisting exclusively of reticulocytes and large ones rich in lymphocytes occurred together and simultaneously (Fig. 3), as opposed to the control. In the majority of follicles relatively rich in lymphocytes, pronounced degeneration and necrosis was observed in the cortex. In the medullary substance, focal degeneration and necrosis was found only occasionally, and these changes were limited to some groups of cells (Figs 3 and 5). In the cytoplasm of mucosal epithelial cells, paranuclear focal necrosis accompanied by the presence of viral particles of helical symmetry also

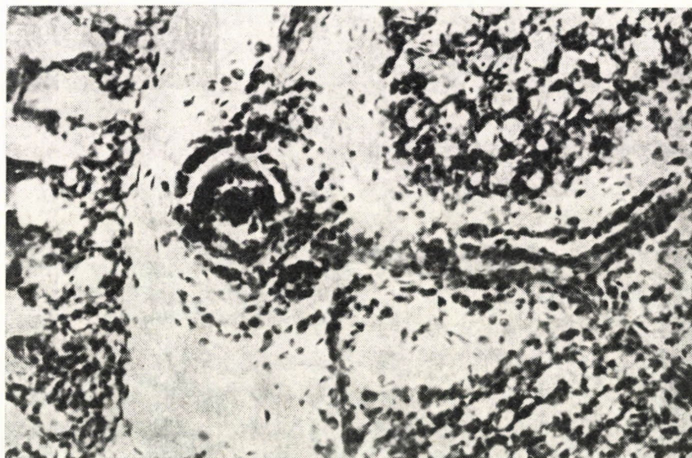

Fig. 1. Detail of lung from a 13-day-old chicken embryo (group A). Groups of granulocytes are seen perivascularly. Haematoxylin-eosin, appr. $\times 160$

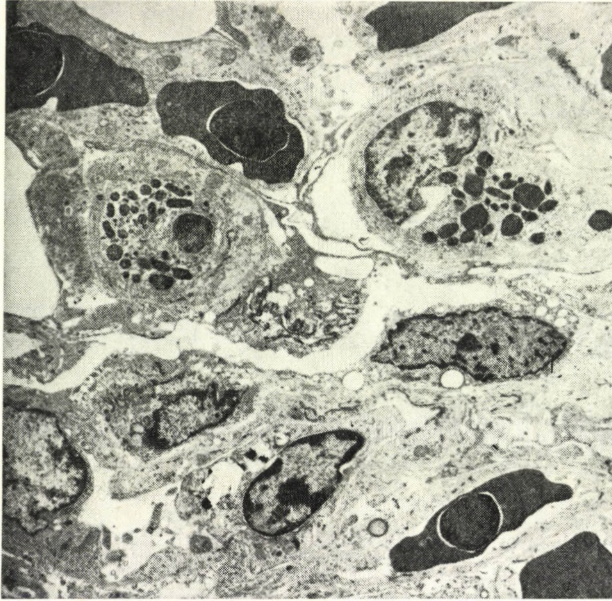

Fig. 2. Detail of lung from a 13-day-old chicken embryo (group A). Heterophil granulocytes can be observed in the interstices. EM ×6400

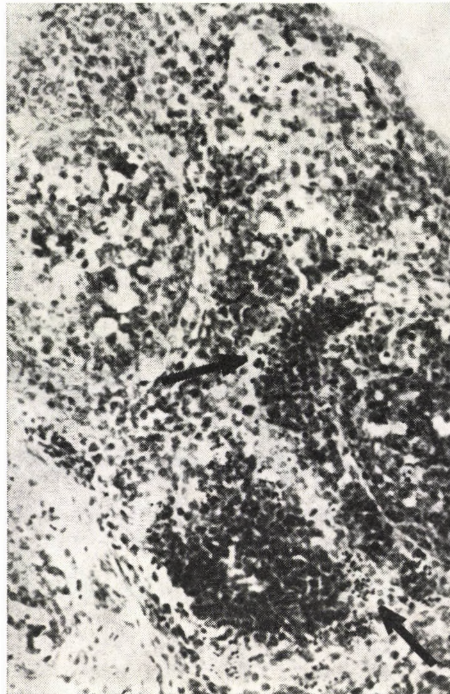

Fig. 3. Detail of bursa of Fabricius from a 18-day-old chicken embryo (group A). As opposed to the control, in infected embryos the rate of lymphocyte colonization in the follicles differs between follicles. Arrow points to disintegration of cells. Haematoxylin-eosin, appr. ×160

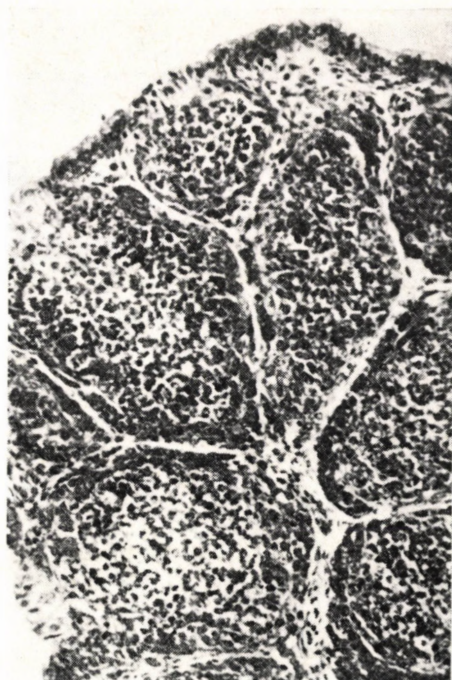

Fig. 4. Detail of bursa of Fabricius from a 18-day-old control chicken embryo. Colonization of lymphocytes in the follicles is uniform. Haematoxylin-eosin, appr. ×160

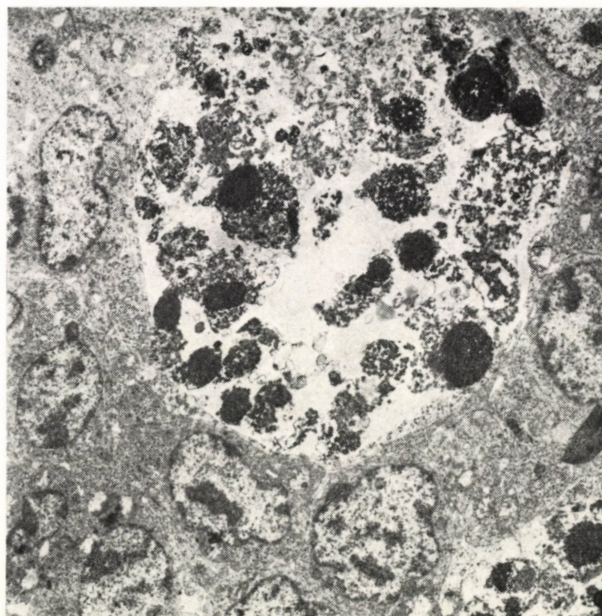

Fig. 5. Detail of bursa of Fabricius from a 18-day-old chicken embryo (group A). Zonal necrosis in the cortex of the follicle. EM ×6400

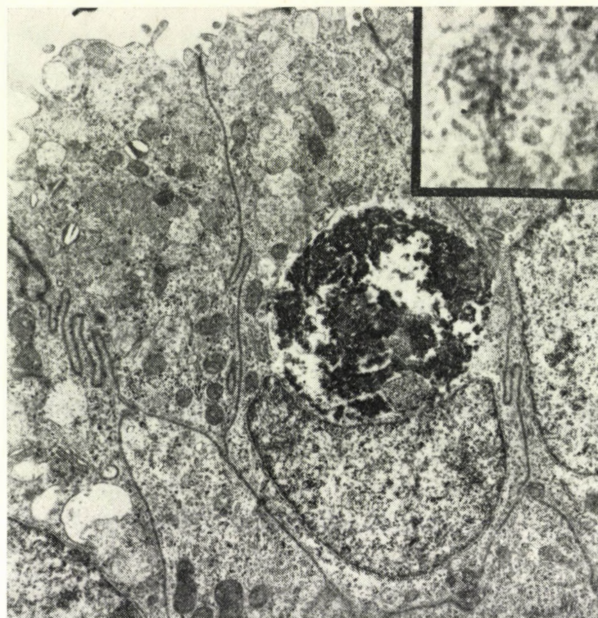

Fig. 6. Detail of bursa of Fabricius from a 18-day-old chicken embryo (group A). Circumscribed paranuclear necrosis of cytoplasm in an epithelial cell of the mucosa. Top right: viral particles in the necrotic area. EM ×14,300; top right: EM ×106,000

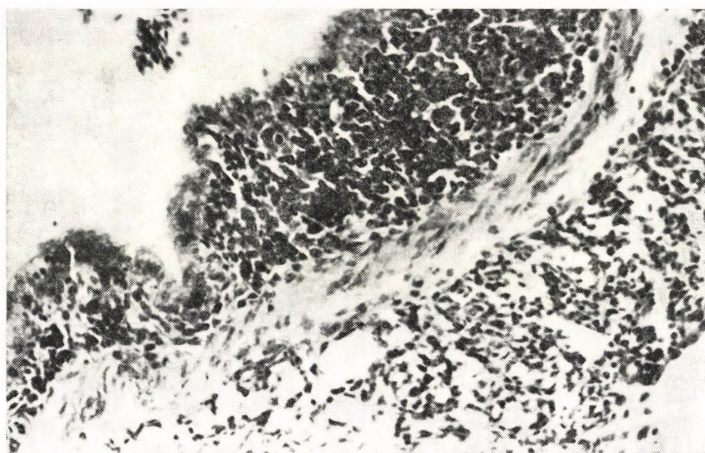

Fig. 7. Detail of lung from a 20-day-old chicken embryo (group A). Infiltration with mononuclear cells in the mucosal membrane of a major respiratory passage. Haematoxylin-eosin, appr. ×160

occurred in some places (Fig. 6). The histological picture of the thymus was similar to that of the controls. In the interstices of the lung and in the walls of major respiratory passages (Figs 7, 8 and 9), and occasionally around the blood vessels and in the connective tissue of other organs (liver, spleen, kidney,

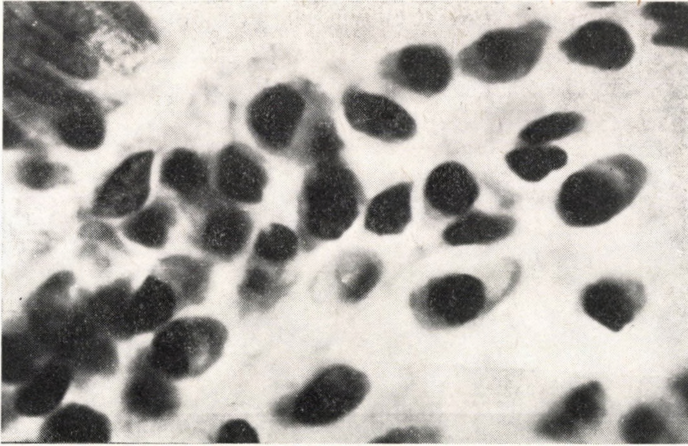

Fig. 8. Detail of lung from a 20-day-old chicken embryo (group A). Plasma cells in the mucosa of the respiratory passage. The mucosa is infiltrated with inflammatory cells. Haematoxylin-eosin, appr. ×1000

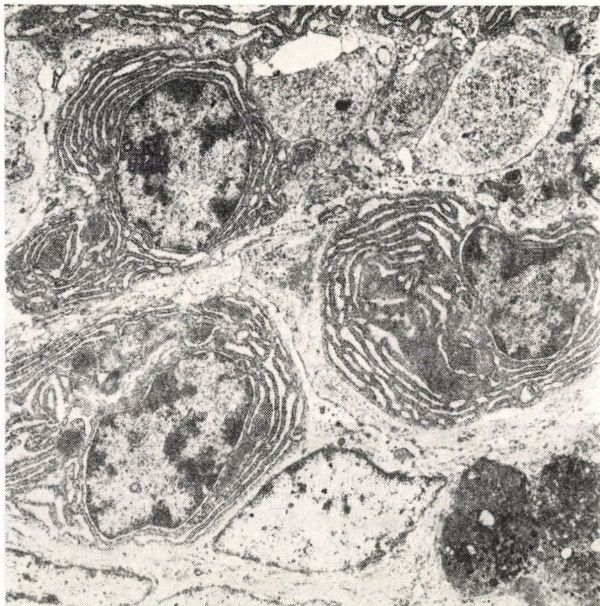

Fig. 9. Detail of lung from a 20-day-old chicken embryo (group A). Plasma cells in the mucosa of the respiratory passage. The mucosa is infiltrated with inflammatory cells. EM ×6400

glandular stomach wall, intestinal wall, heart, central nervous system), mononuclear cells (lymphocytes, lymphoblasts, histiocytes and plasma cells) were found to occur in groups or dispersed. On the 10th day after hatching, chicks of this group showed hyperplasia of lymphoid tissues or organs (i.e. presence of more and larger follicles rich in lymphoblasts than in the controls) and tissue plasmacytosis.

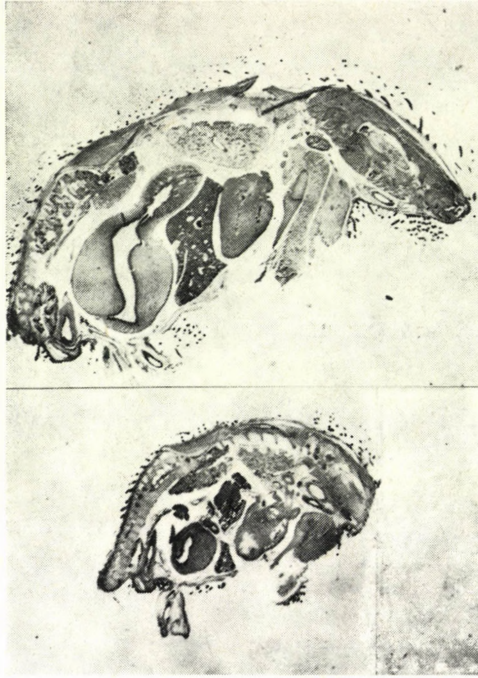

Fig. 10. Longitudinal section of chicken embryos in the plane of the vertebral column. Above: control chicken embryo; below: chicken embryo infected with B_1 virus on the 6th day of incubation. Haematoxylin-eosin, natural size

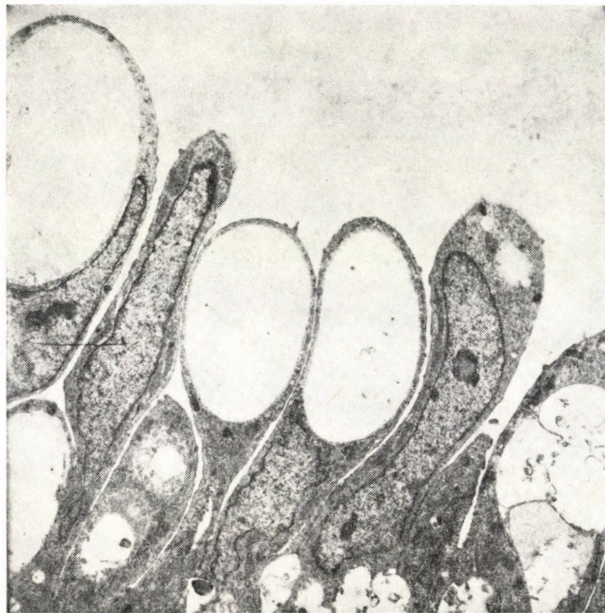

Fig. 11. Detail of lung from a 5-day-old chick (group C). The epithelium of the bronchial mucosa shows cystic degeneration of varying severity. EM ×9600

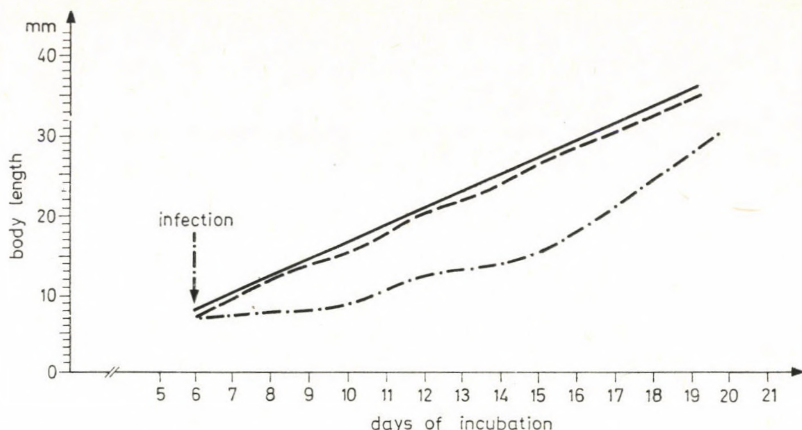

Fig. 12. Growth of chicken embryos infected with B_1 virus (——— control; -·-·- embryos infected on 6th day of incubation; - - - embryos infected on 13th day of incubation)

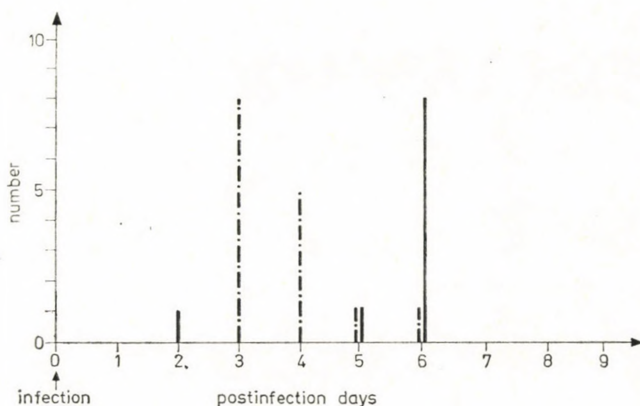

Fig. 13. Mortality of chicken embryos infected with B_1 virus (-·-·- mortality of chicken embryos infected on the 6th day of incubation; ——— mortality of chicken embryos infected on the 13th day of incubation)

The average body size of embryos belonging to group A (the length of trunk of embryos sectioned longitudinally in the plane of the vertebral column) was considerably smaller than that of group B embryos and controls, up to the time of hatch-out (Figs 10 and 12). The time and rate of mortality are indicated in Fig. 13.

Bacteriological and mycological examination of the yolk sac cavities, livers and of the liver and bone marrow of hatched chicks gave negative results. In the HI test, group A chicks bled on the 10th day after hatching gave the lowest mean titres (Table I).

In the second half of incubation, *group B* embryos showed tissue changes similar to those found in group A embryos of the same age. However, in each of group B embryos, bronchopneumonia also occurred.

Table I

Results of HI tests in chicken embryos (groups A and B) and day-old chicks (group C) infected with B_1 virus

Groups	A	B	C	
	1 : 2	1 : 8	1 : 2	1 : 8
	1 : 2	1 : 2	1 : 16	1 : 16
	1 : 2	negative	1 : 64	1 : 8
	1 : 2	negative	1 : 4	1 : 32
	negative		1 : 16	1 : 8
			1 : 8	1 : 4
			1 : 32	1 : 2
			1 : 8	
Mean titre	1 : 1.6	1 : 2.5	1 : 15.2	

The average body size of group B embryos did not differ significantly from that of the controls. Bacteriological and mycological examination of the yolk sac cavities, livers and lungs, and of the liver and bone marrow of hatched chicks gave negative results in all cases. The HI titres obtained for group B chicks bled on the 10th day after hatch-out only slightly exceeded those found for group A birds.

The changes observed in chicks of *group C* (infiltration with mononuclear cells in different organs, plasmacytosis) were similar to those found in survived chicks of groups A and B after hatch-out. In addition to these, in several cases vacuolar degeneration was seen in epithelial cells of the infiltrated mucosa of respiratory passages (Fig. 11). On the other hand, hyperplasia of the follicles of lymphoid organs and tissues was not accompanied by notable lymphocyte

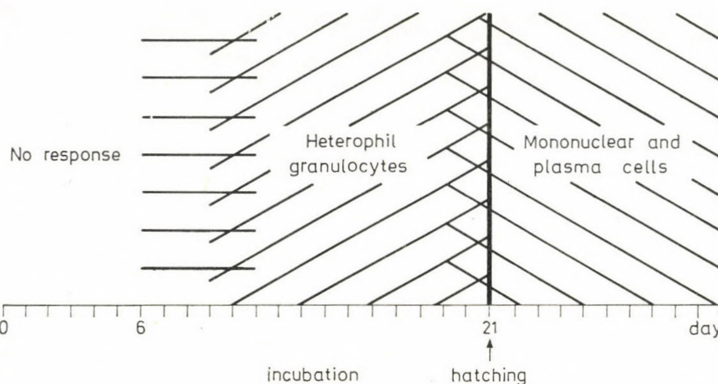

Fig. 14. Types of cellular reactions in chicks and chicken embryos infected with B_1 virus

degeneration. No mortality was observed in this group. Mean HI titres of group C chicks bled on the 10th day after hatching significantly exceeded those found for group A and group B chicks.

The time of appearance of virus-induced cellular and tissue reactions and the change of their character in embryos and chicks of the three groups are illustrated in a scheme (Fig. 14).

Discussion

According to the present results, chicken embryos and day-old chicks of different ages gave diverse responses to infection with the B_1 virus strain. *In the first phase*, up to the 10th day of incubation, the death of infected embryos occurred most frequently on the 3rd day postinfection. The gross pathological examination revealed oedema and haemorrhages in different organs of the dead embryos. These lesions indicated that the rapid death of young embryos could have been due to the B_1 virus-induced circulatory disturbances and damages in the walls of blood vessels. Of all experimental groups, the mean values of specific antibody titres were lowest in the group infected in this phase of incubation. The development of infected and survived embryos was significantly retarded, as compared to embryos infected in later phases of development and to controls.

In the second phase, in embryos examined between the 10th and 19th day of incubation, mortality was highest on the 6th day postinfection, and heterophil granulocytic infiltration was observed in some organs of the examined embryos. In the opinion of Delaney and Ebert (1962), in chicken embryos the colonization of lymphocytes in the follicles of the bursa of Fabricius is not completed by the 11th—19th day of incubation. According to Szenberg (1977) and our own observations (Glávits et al., 1982), in this period intensive granulocytopoiesis takes place in the spleen and bursa of Fabricius of the embryos. In all probability, this plays a part in the development of heterophil granulocytic infiltration due to B_1 virus infection in embryos infected between the 10th and 19th day of incubation. In the second phase of incubation, paranuclear focal cytoplasm degeneration and necrotic areas containing viral particles were also demonstrable in mucosal epithelial cells of the respiratory tract and bursa of Fabricius of the embryos. B_1 virus has already been reported to cause damage in lymphoid tissues (Tizard, 1977; and others). In embryos examined in the second phase, mean antibody titres were slightly higher than in embryos examined in the first phase. The development of infected but survived embryos did not fall behind that of the controls.

In individuals examined between the 19th day of incubation and the 10th day after hatch-out *(third phase)*, mean titres of specific antibodies

significantly exceeded those of embryos infected in earlier phases. Among chicks infected in the third phase, neither disease nor mortality occurred. The histological and electron-microscopic examination showed marked infiltration with mononuclear cells in the organs of embryos, which was the tissue response to the B_1 virus infection.

The observations of certain authors (Firth, 1977; Glick, 1979) and our own experiments indicate that in chicken embryos the normal development of peripheral lymphoid organs and tissues, as well as the complete colonization of lymphocytes in the bursa of Fabricius, are finished by the 19th day of incubation. Therefore, the weaker effect of B_1 virus infection in third-phase embryos may be attributed to the more advanced developmental status of lymphoid organs.

The development of the immune responsiveness is indicated by the hyperplasia observed in lymphoid organs. In conclusion, our results show that the humoral immune responsiveness of chicken embryos and day-old chicks to B_1 virus and the types of pathological lesions induced are determined by the developmental status of the lymphatic and myeloid systems at the time of infection. In their experiments with different viruses and other pathogenic agents in chicken embryos and day-old chicks, several authors have arrived at the same conclusion (Woodruff and Goodpasture, 1931; Koprowski and Cox, 1947; Loomis et al., 1950; Scheidegger, 1953; Chute and Cole, 1954; Love and Roca-Garcia, 1955; Casorso and Jungherr, 1959).

Finally, our observation that in chicken embryos infected with the B_1 virus strain in an early phase of embryonic development, the development of organs of the immune system is impaired and takes place at a slower rate, is remarkable. This is of great practical significance, since later on the damaged immune system may have an adverse influence on the efficiency of immunizations and on the outcome of post-hatching infections.

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STUDIES ON THE IMMUNOLOGICAL ADJUVANT ACTIVITY OF *BORDETELLA BRONCHISEPTICA* IN MICE

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The immuno-adjuvating effect of *Bordetella bronchiseptica* strains of different origins was studied. The main target of the experiment was to find an immunological adjuvant exhibiting at least as high degree of immunoadjuvancy as *B. pertussis*, widely applied in human immunization practice as well as in laboratory trials. As test antigen, adsorbed tetanus toxoid was administered. Using the active mouse-immunization–toxin challenge test for the determination of adjuvating properties of the investigated *Bordetella* strains we concluded that (i) the majority of the *B. bronchiseptica* strains significantly increased the antigenicity of adsorbed tetanus toxoid. Depending on the applied amount of the *B. bronchiseptica* adjuvants, the protectivity increased 1.5—3 times; (ii) on the basis of dose-dependency and adjuvating property we failed to find any difference between the adjuvating mechanism of the two *Bordetella* species.

Keywords. *Bordetella bronchiseptica*, immunomodulation, tetanus toxoid.

Vaccines of many Gram-negative bacteria greatly enhance the antibody production to unrelated antigens if administered to animals or man at the same time. The best-known example is the increased potency of diphtheria and tetanus toxoids when combined with pertussis vaccine. Greenberg and Fleming (1947, 1948) observed the adjuvant effect of *Bordetella pertussis* vaccine on the response to diphtheria toxoid in guinea-pigs, and Fleming et al. (1948) obtained similar results in children. Later *Bordetella parapertussis* was proved to be an immunological adjuvant of high value, similarly to *B. pertussis*. The whole-cell suspension prepared from *B. parapertussis* efficiently enhances the immune response to tetanus toxoid in both experimental animals and humans, amplifies the immunological potential, thus it can be used effectively for overcoming the low-intensity immune response, due to antigenic competition. The *B. parapertussis* suspension exhibits this effect independently of being applied alone or associated to *B. pertussis* suspension.

Since the practical implications of this phenomenon are being used in human vaccine production on a large scale (Faragó and Pusztai, 1949; Joó and Réthy, 1957), we set before us as an object to investigate the possibility of applying *Bordetellae* as antigenic adjuvants in veterinary practice. In the

present experiments we attempted to determine whether *B. bronchiseptica* causing respiratory disorders in several animal species can be used as an immunological adjuvant. The effect of inactivated *B. bronchiseptica* suspensions of different origins on the immune response to tetanus toxoid was studied.

Materials and methods

Origin of B. bronchiseptica

The eight *B. bronchiseptica* strains were isolated from the nasal cavities of pigs in four herds at different locations. The herds did not show apparent clinical symptoms of atrophic rhinitis.

Culture media applied for isolation

B. bronchiseptica was isolated by inoculating nasal swab samples on modified MacConkey's agar containing 1% dextrose. After 48 h of incubation at 37 °C, *B. bronchiseptica* appeared on this medium as greyish-tan colonies. The identity of the bacterium was confirmed by biochemical tests as described by Pittman (1974) in Bergey's Manual and by tube agglutination tests with hyperimmune antisera prepared in rabbits by seven intravenous injections of live *B. bronchiseptica*. Each strain was kept in freeze-dried form in presence of dextran (MW 50,000), and propagated on 5% sheep blood agar.

Preparation of whole-cell suspensions

B. bronchiseptica cultures incubated at 37 °C for 48 h on 5% sheep blood agar medium in Petri dishes were suspended in phosphate-buffered saline (PBS), and the suspensions of eight strains were adjusted to concentrations of both 10 and 30 International Opacity Units (IOU) per ml as compared to the International Opacity Standard issued by National Institutes of Health (NIH), Bethesda, Md., USA.

The organisms were inactivated by the addition of 1 : 5000 Merthiolate.

B. pertussis suspensions

As control, inactivated *B. pertussis* suspensions (designated PE-1, PE-2 and PE-3) were also applied on the basis of their IOU content. The *B. pertussis* suspensions, containing 330 IOU per ml, were prepared by the Vaccine Laboratory of the HUMAN Institute.

Tetanus toxoids

Adsorbed tetanus toxoid was used in two different forms: (i) as purified tetanus toxoid adsorbed on 4.5 mg of aluminium phosphate per ml (1 mg $Al^{\cdots}$ per ml), containing 10 Binding Units (BU) of tetanus toxoid; (ii) native adsorbed tetanus toxoid, used in veterinary practice. In this case aluminium hydroxide was the carrier, and 10 BU of tetanus toxoid was adsorbed per ml of the final vaccine.

Experimental procedure

The whole-cell suspension was first inactivated with Merthiolate, adjusted to the appropriate IOU value (WHO), associated with the undiluted adsorbed tetanus toxoid, and twofold serial dilutions of the control toxoid and toxoid + adjuvant mixture were made.

Each dilution was used for subcutaneous immunization of 16 albino mice weighing 18 g each. The immunizing dose was 0.5 ml per mouse in each case.

The immunized animals were challenged with 10 Minimal Lethal Dose (MLD) tetanus toxin on the 21st day following immunization. The proportion of animals died with specific symptoms was registered during a five-day observation period.

Subsequently, the 50% effective doses (ED_{50}) and the virtual protective effect (in International Immunizing Units (IIU)) of the immunizing mixture or monovalent tetanus toxoid, as compared to that of the International Standard Tetanus Toxoid, were calculated (Hegedüs and Réthy, 1967).

Results

The results are summarized in Table I. The data show that whole-cell suspensions prepared from both inactivated *B. pertussis* and *B. bronchiseptica* have an immunological adjuvant effect of high degree in case of joint application with tetanus toxoid.

All the 3 examined *B. pertussis* and 8 *B. bronchiseptica* strains showed well-defined dose-dependency, i.e. the application of 30 IOU induced higher degree of immunoenhancement as a rule than 10 IOU. This dose-dependency was significant in all cases, with the exception of strain CF. A further argument of the dose-dependent adjuvating activity of *Bordetellae* is the fact that two *B. bronchiseptica* strains (B 21, C 42) and one *B. pertussis* strain (PE 2) failed to enhance the efficacy of the tetanus toxoid if applied in amounts corresponding to 10 IOU. At the same time all three *Bordetella* strains

Table I

Examination of the dose-dependent immunological adjuvant effect of inactivated *Bordetella* bacterial suspensions

Adjuvant dose	NO	10 IOU	30 IOU
Efficiency of tetanus toxoid		IIU per ml	
Designation of *B. bronchiseptica* strains			
B 5	51	107++	300+++
BAK	51	190+++	239++
BÁB	51	90.3+	261+++
DK 2	51	127++	238+
B 21	51	52	104+
C 42	51	49	110++
CE	150	226++	350++
CF	150	201 NS	242 NS
B. pertussis			
PE 1	150	238++	334+
2	51	61.5	109.5+
3	51	169++	239++

Statistical significance of the degree of dose-dependent immunoenhancement
+P = 0.005 ++P = 0.002 +++P = 0.001
NS = not significant
Active immunization–toxin challenge test in mice
Antigen: adsorbed tetanus toxoid 10 BU per ml as follows:
51 (39–68) IIU per ml = immunizing value of purified tetanus toxoid adsorbed on aluminium phosphate; in brackets: 95% fiducial limits
150 (108–207) IIU per ml = immunizing value of native tetanus toxoid adsorbed on aluminium hydroxide

adjuvated the antigenicity of the test antigen in amounts corresponding to 30 IOU.

All of the examined 11 strains were individually shown to possess different immunostimulant properties. In this respect outstanding values were obtained for strains PE 1 and CE, while strains designated B 5 and BÁB were found to show the most prominent dose-dependency.

Discussion

The excellent immunostimulant properties of *B. pertussis* have long been known and are being exploited in human medicine, mainly in the combined immunization of children against diphtheria, tetanus and pertussis with a

combined vaccine to prevent the antigenic competition between tetanus and diphtheria toxoids.

In the present experiments we investigated the veterinary medical aspects of the problem, and attempted to determine whether *B. bronchiseptica* strains possess similar activity. The immunological enhancing (immunomodulating) effect of the isolates was determined in "screening experiments", using adsorbed tetanus toxoid as test antigen. The immunological adjuvating effect was judged on the basis of the degree of increase in the antigenicity of tetanus toxoid. So this procedure enabled us to select those strains exhibiting high adjuvating activity, strains which in the future possibly can be applied in veterinary medical practice.

The above results demonstrate that the examined *B. bronchiseptica* strains showed an immunological adjuvant effect equivalent to that of *B. pertussis* strains. The results obtained with the 8 strains examined so far indicate that there are considerable differences in the immunostimulant capacity of the individual strains. Furthermore, the immunological adjuvant effect showed a pronounced dose-dependency, which was significant for all but one of the strains.

Further studies are needed to determine the bacterial cell component responsible for the immunological adjuvant effect.

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SOME FACTORS INFLUENCING THE IMMUNOLOGICAL ADJUVANT EFFECT OF *BORDETELLA BRONCHISEPTICA* ON THE IMMUNE RESPONSE TO TETANUS TOXOID

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The immunological adjuvant effect of killed *Bordetella bronchiseptica* bacterium suspensions was studied in immunization experiments with tetanus toxoids of different degrees of "intrinsic antigenicity".

Dose-dependent adjuvating effect of *B. bronchiseptica* suspensions was demonstrated in immunization trials with the different tetanus toxoids. In case of immunization with the toxoid of higher intrinsic antigenicity, the degree of adjuvating effect was lower than in case of application of the tetanus toxoid of lower intrinsic antigenicity.

Comparative studies on mice stocks of low and high immuno-potential revealed that low-responder mice could be immunized efficiently neither with toxoid alone nor with that associated with the adjuvant prepared from *B. bronchiseptica*.

Keywords. *Bordetella bronchiseptica*, immunomodulation, intrinsic antigenicity, immuno-potential.

We have recently, to the best of our knowledge first in the literature, described that *Bordetella bronchiseptica* has an excellent immunological adjuvant effect regarding the development of the anti-tetanus immunity (Réthy et al., 1982*a*).

Thus, a further bacterial species has been found to show the long-known phenomenon that whole-cell suspensions (bacterial vaccines) of certain, mainly Gram-negative, bacteria can greatly enhance the immune response to unrelated antigens (Ramon, 1925).

A relatively large series of experiments was aimed at defining the main immunological (immunoadjuvant, immunomodulant) features of immunostimulants: in the first line to investigate possibility of applying them in human and/or veterinary immunization practice (Géresi et al., 1982; Réthy et al., 1982*b*). The main goal of our present paper was to get replies to the following questions:

(i) To what extent, if at all, can the supplementary immunoadjuvant effect influence the development of the primary anti-tetanus immunity if the

two main representatives of tetanus toxoid, i.e. tetanus toxoid of low intrinsic antigenicity and the one of high intrinsic antigenicity (Dresser and Mitchison, 1960), are administered as test antigen.

(ii) To what extent can the *B. bronchiseptica* whole-cell immunomodulant influence the genetically defined immuno-potential or the immunological potential suppressed by environmental factors. The main question in this field was whether *B. bronchiseptica* can, or cannot, replace the excellent immunomodulant, T-helper-replacing, immuno-amplifying effect of lipopolysaccharides (Réthy, 1981; Géresi and Réthy Jr., 1982).

Materials and methods

Inactivated B. bronchiseptica suspensions

B. bronchiseptica strains B 5 and CE were used. These strains showed the highest immunostimulant effect during the previous study. The strains were kept freeze-dried in presence of dextran and propagated on 5% sheep blood agar plates.

The preparation of suspensions and inactivation of microorganisms were performed as described recently (Magyar et al., 1983; see p. 31 in this volume), with the exception that the density of the suspension prepared from *B. bronchiseptica* strain CE was adjusted to 30 IOU only. (IOU = International Opacity Unit, defined against the International Opacity Standard issued by the National Institutes of Health, Bethesda, Md., USA under auspices of World Health Organization).

Tetanus toxoids

Two tetanus toxoids were administered as aluminium-carrier-adsorbed monovalent vaccines throughout the experiments. The two toxoids represented the two main antigenicity groups of tetanus toxoids, i.e. (i) one toxoid belonged to the group of low intrinsic antigenicity (Toxoid I), while the other to (ii) the group of high intrinsic antigenicity (Toxoid II). The detailed description can be found under Results and discussion.

The adsorbed toxoids, irrespectively of their antigenicity characteristics, contained equally 10 BU (Binding Units) per ml of final vaccine.

Mice

Two groups of mice — from different breedings — were applied for defining the immunological-potential-increasing activity of *B. bronchiseptica* vaccines.

Mice of group no. 1 exhibited fairly sensitive immunological potential as checked with increasing doses of the test antigen. If combining lipopolysaccharide to the test antigen, the immune response increased significantly and uniformly in all individuals.

Mice deriving from the other breed exhibited poorly-reacting immunological potential. A certain part of the investigated animals behaved like "non-responders", in spite of increasing the dose of antigen. Lipopolysaccharide deriving from *Shigella sonnei* significantly decreased the proportion of non-responders (Géresi, 1981).

Experimental procedure

Determination of the potency, and also of the changes in potency, while applying immunomodulants was performed in mouse active immunization test followed by challenge with 10 Minimal Lethal Dose (MLD) of tetanus toxin per mouse. Both the immunization and the toxin-challenge were performed by the subcutaneous route. A single injection of 0.5 ml was given throughout. The immunization procedure has been described in detail by Hegedüs and Réthy (1967), and the presently applied form of defining the changes in potency using the mentioned mouse active immunization–toxin challenge test has been described in the present volume by Réthy et al. (1983) (see p. 43).

Results and discussion

Investigations concerning the immunological characteristics of the test antigens

During the experiments aimed at defining the "supplementary" adjuvating effect of the *B. bronchiseptica* immunomodulants exerted on the tetanus toxoids' effectiveness determined by the degree of intrinsic antigenicity, we investigated the characteristics of the tetanus toxoids (Table I).

Table I displays the main characteristics influencing the "own" antigenicity of tetanus toxoids.

Toxoid II (non-purified), investigated with double diffusion test and Oudin's semisolid gel-precipitation method against hyperimmune tetanus antitoxin showed as many arcs and bands as the purified toxoid designated as Toxoid I.

We neither were able to find the reason of the higher antigenicity of Toxoid II regarding other parameters of the two toxoids investigated.

So, we had to accept the theorem launched by Dresser and Mitchison (1960) about the different degrees of intrinsic antigenicity values of antigens.

This theorem was, however, modified by one of us based on his own experience on tetanus toxoids' antigenicity. He formulated the dose–response

Table I

Immunologically relevant parameters of the tetanus toxoids applied throughout the experiments

	Purity degree BU/ml	No. of arcs+ and bands++ in		IIU□ per BU	IU+++ in guinea-pig test
		Ouchterlony test	Oudin test		
Toxoid I	1400	10	11	9.5	4.8
Toxoid II	600	11	11	21.75	5.8

Remarks:

+ and ++: immunodiffusion test according to Ouchterlony and Oudin (see: respective handbooks);

□ antigenicity, as determined in mouse active immunization–toxin-challenge test. P between Toxoid I and Toxoid II results 0.001;

+++Antigenicity, as determined according to the "one stimulant method" (see: any Pharmacopoeia; Monograph on tetanus toxoid).

function concerning the tetanus and diphtheria toxoids. Using the equation, we can express the degree of the intrinsic antigenicity by the value of the "ordinate-intercept" of the equation (Réthy, 1981).

Experiments with immunomodulants and tetanus toxoids

Using as test antigens the above-described antigens, the following results were achieved:

Table II

Immunological adjuvant effect of inactivated *B. bronchiseptica* suspensions on the immune response to tetanus toxoids of different intrinsic antigenicity

Toxoid	Toxoid I		Toxoid II		
B. bronchiseptica strain B 5	0	10 IOU	0	10 IOU	30 IOU
ID/mouse	Mortality in percentage of immunized mice				
1 : 30	—	—	—	—	—
1 : 60	19	—	—	—	—
1 : 120	27	12	7	—	—
1 : 240	75	53	13	12	7
1 : 480	100	81	81	54	37
1 : 960		100	100	100	87
1 : 1920					100
IIU/ml	95	158	217	276	350

Active protection–toxin challenge test in mice
Antigen: adsorbed tetanus toxoid, 10 BU/ml
ID = immunizing dose, in ml
IIU = International Immunizing Units

Table II shows the immunological adjuvant effect of the suspension prepared from inactivated *B. bronchiseptica* strain B 5, during immunization with tetanus toxoids with intrinsic antigenicity of varying degree. Table II contains the complete record of the experiment to illustrate the significant differences existing in immunogenicity between the purified antigen of low intrinsic antigenicity and the native antigen of higher intrinsic antigenicity. When using antigens of identical origin, the immunogenicity of the native tetanus toxoid is 2.5 times higher than that of the purified antigen prepared from the former. While 10 IOU of the adjuvant elicited a twofold increase in the immunogenicity of the purified antigen of lower efficiency, 30 IOU of the immunological adjuvant were needed for inducing a significant increase in the efficiency of the native antigen, which had already been highly effective originally. This phenomenon can unanimously be traced back to the restricted immunological potential of the experimental animals (mice), and to the possible maximum activation of this potential.

This view seems to be supported by the experiment in which the immunomodulant (immunological adjuvant) effect of the *B. bronchiseptica* suspension was studied in mice originating from two different strains (Table III).

Table III

Immunological adjuvant effect of inactivated *B. bronchiseptica* suspension in groups of mice of different immunological potential

B. bronchiseptica strain CE	Group no. 1 of mice		Group no. 2 of mice	
	0	30 IOU	0	30 IOU
ID/mouse	Mortality in percentage of immunized mice			
1 : 30	—	—	25	12.5
1 : 60	—	—	20	37.5
1 : 120	6.7	—	38	62.5
1 : 240	13.3	7.1	75	68.7
1 : 480	81.2	37.5	100	93.8
1 : 960	100.0	87.5		100.0
1 : 1920		100.0		
IIU/ml	216	350	156.1	149.4
95% fiducial limit	81%–123.7%	79.4%–125.8%	60%–180%	60%–180%

It was proved in earlier experiments (Géresi, 1981) that experimental mice strain no. 2 cannot be used for the determination of biological variables because mice of this group develop poor protection to antitoxigenic antigens. In the present experiment we attempted to modulate the immunological potential of mice with the suspension of *B. bronchiseptica* CE strain, which had proved to be the most efficient in earlier experiments.

Table III contains results of a comparative study. In this experiment, mice derived from two different stocks were immunized with the same adsorbed tetanus toxoid, and immunomodulation with 30 IOU of the *B. bronchiseptica* strain CE was performed in the same way in both groups of mice.

The observations have unanimously confirmed our earlier hypothesis that the majority of mice belonging to group no. 2 (i) cannot be immunized with antitoxigenic antigens at all, i.e., most of them fail to develop antitoxic protection even to high doses of toxoid; and (ii) that in this group of mice the *B. bronchiseptica* suspension used as immunological adjuvant did not amplify the immunological potential in the majority of immunized animals. This is proved by the fact that protection or lack of protection is of identical degree practically at all immunization-dose levels in group no. 2. The 95% fiducial limits obtained for the immunized population were significantly broader than in group no. 1.

In conclusion, we can establish that, in accordance with the principles of immunomodulation, the immunostimulant effect of immunological adjuvants depends on the immuno-potential of experimental animals, and on the extent to what it can be activated. This is suggested by our results obtained for experimental animals representing different immunological potentials, and in experiments on enhancing the efficiency of tetanus toxoids of varying intrinsic antigenicity.

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IMMUNOMODULATION OF PRIMARY ANTITOXIC-TYPE IMMUNITY WITH INACTIVATED *BRUCELLA* SUSPENSIONS

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Killed whole-cell *Brucella abortus* and *B. suis* suspensions were applied as adjuvants in active mouse-protection tests with tetanus and *Cl. perfringens-D* (epsilon) toxoids. The *Brucella* suspensions exhibited high degree of adjuvancy in simultaneous application with either of the toxoids. The increase in antigenicity ranged between 105 and 484%. The numerical data got by the application of a polynomial equation were processed with an analysis of variance. The results in repeated adjuvant trials concerning the effectiveness of both toxoids showed identity. Computed from comparative experiments with the two toxoids, the values of the total-determination-coefficient ($R^2 = 0.91$ at $P = 0.01$), and those of the determinant-coefficient of linearity ($r^2_{lin} = 0.83$ at $P = 0.01$) show a strict parallel run of the two curves representing the results got with the two toxoids.

Keywords. Immunomodulation, primary immunity, inactivated *Brucella.*

In a series of experiments we defined the immunomodulating activity of several substances of biological origin. The experiments were performed as laboratory screening-type trials applying the following immunological in vivo models:

(i) in experiments aimed at the definition of the immunomodulant activity of the investigated substances exerted on the development of humoral (antitoxic) immunity, active mouse-immunization followed by toxin challenge was applied;

(ii) the immunopotentiating activity exerted on the development of antibacterial immunity was investigated with the use of *Erysipelothrix rhusiopathiae* vaccine;

(iii) the host-defence-increasing/modifying activity of the substances was determined in mouse-tumour systems, applying Ehrlich's ascites tumour and mouse fibrosarcoma No. S-180.

The present experiments were aimed at defining the immunomodulating activity of heat-inactivated *Brucella* suspensions on the development of primary antitoxic immunity. The main goal of the experiments was to get know some features of the adjuvating activity of *Brucella* suspensions exerted on

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the development of the anti-tetanus and anti-*Cl. perfringens-D* (epsilon) primary protection in comparative trials.

We have shown recently that inactivated *Brucella* suspensions have immunomodifying effect on the host defence or on the development of antitoxic or antibacterial immunity (Toujas et al., 1972, 1975; Réthy et al., 1980; Géresi et al., 1981).

Materials and methods

Brucella strains were cultured on potato-agar medium. The harvest was inactivated by heat at 60 °C and suspended in phosphate-buffered saline. The strains are listed and their relevant characteristics are displayed in Table I.

Table I

Characteristics of the inactivated *Brucella abortus* and *B. suis* suspensions applied in the experiments

Origin of immunomodulators+	Inactivated bacterium suspensions		
	IOU per ml++	Dry weight per ml, in mg	IOU per mg dry weight
Brucella abortus PH-0048	570	26.69	21.35
Brucella abortus PH-0159	690	39.80	17.34
Brucella abortus PH-0260	480	23.34	20.57
Brucella abortus PH-0371	570	27.18	20.97
Brucella suis PH-0482	410	21.41	19.15
		Average:	19.876
		i.e.: 20 IOU per 1 mg	

Remarks:

+ Numbers of the strains are given as registered in PHYLAXIA.

++ IOU = International Opacity Unit, as compared to the NIH—WHO Opacity Standard.

The bacterial count was adjusted against the International Opacity Standard issued by the National Institutes of Health and accepted by the World Health Organization. So, all the data concerning the amounts applied are expressed in terms of International Opacity Units (IOU).

Determination of the potency of tetanus and Cl. perfringens-D (epsilon) toxoids

Standards. The National Substandard Tetanus Toxoid Preparation contained 50 International Immunizing Units (IIU) in freeze-dried form per ampoule. The local standard for *Cl. perfringens-D* (epsilon) toxoid equalled to 43 local protective units (PU) per ml, in aluminium-hydroxide-adsorbed form.

Experimental procedure. The potency-determinations of tetanus toxoid and *Cl. perfringens-D* (epsilon) toxoid were performed uniformly in mouse

groups, using the active mouse-immunization–toxin challenge test. This test was described in essence by Hegedüs and Réthy (1967). The method, applied for the *Cl. perfringens-D* (epsilon) toxoids' potency assay was evolved by Géresi (1981).

(i) Active mouse-immunization–toxin challenge test

Twofold serial dilutions were made of both toxoids, using *Brucella* suspensions containing 4.0 IOU per ml as diluent. The single immunizing dose — at each dilution level — contained 2.0 IOU of immunomodulant suspension besides the diluted adsorbed toxoid. Controls received toxoid only.

Groups of 20 albino Swiss mice were formed at random. One-shot immunization was performed by the subcutaneous route with 0.5 ml of the mixtures mentioned above (See preceding paragraph).

Toxin challenges were performed on the 21st day after immunization. The tetanus toxin, 20 Minimal Lethal Dose (MLD) per animal was administered subcutaneously, whereas the *Cl. perfringens-D* (epsilon) toxin, 3 MLD/animal, intravenously.

(ii) Computation of relevant data of the tests

Using the 50% protective dose-values, characteristic of test and control groups, the immunomodulator treatment-specific Relative Potency (RP) and the changes of immunizing values under the interaction of *Brucella* immunomodulators expressed in IIU or, in case of *Cl. perfringens* toxoid, in local PU, were also determined and 95% fiducial limits of the RP values were defined.

(iii) All the experiments were performed twice at a relatively long interval. This was done to define the reproducibility of results and to make the immunological data more precise, thus suitable for investigating parallelism between the data concerning the two toxoids under the influence of different *Brucella* immunomodulators. To define and statistically analyse the identity of results, the proportionality expressible in numerical data of biological interactions, the non-linear regression analysis was applied, followed by the analysis of variance and definition of determination-coefficients computed by the use of the polynomial equation, i.e. $Y = a + bx + cx^2$ (Sváb, 1981).

Results

The effect of *Brucella* immunomodulant treatment on the development of primary anti-tetanus (antitoxic) immunity, i.e. the effect exerted on the development of the protection against tetanus toxin intoxication is presented in Table II.

The protective value of the tetanus toxoids increased under the influence of the application of immunomodulants prepared from different *Brucella* strains 1.54–4.84 times.

Table II

The development of primary anti-tetanus protection and the effect of immunomodulators prepared from different *Brucella* strains. Examination of the reproducibility of results using analysis of variance

Origin of immunomodulators+ (Dose: 0.1 mg)	Relative potency results of the repeated investigations with 95% fiducial limits	
	Investigation I++	Investigation II++
Brucella abortus PH-0048	2.98 (2.35–3.78)	3.16 (2.59–3.86)
Brucella abortus PH-0159	3.21 (2.54–4.08)	3.42 (2.80–4.17)
Brucella abortus PH-0260	1.94 (1.53–2.46)	2.01 (1.65–2.45)
Brucella abortus PH-0371	4.84 (3.82–6.15)	4.29 (3.52–5.23)
Brucella suis PH-0482	1.54 (1.21–1.96)	1.76 (1.44–2.15)
Untreated control	1.0 (0.79–1.27)	1.0 (0.82–1.22)

Analysis of variance: $R^2 = 0.96$ $P < 0.01$; $r^2_{lin} = 0.95$ $P < 0.01$

Source	SQ	DF+++	MQ	$F_{P\,=\,0.01}$
Total	8.9654	5		
Regression	8.6126	2	4.3643	36.62 > 30.38
linear.	8.5245	1	8.5245	72.48 > 34.12
square	0.0881	1	0.0881	not significant
Error	0.3528	3	0.1176	

+ See footnote to Table I
++ Interval between the two investigations: six weeks
+++ DF = degree of freedom

The data of the repeated investigations show an excellent reproducibility. Analysis of variance shows a strict parallel run of the data, independently of the time of the investigation.

The data of Table III show the interaction of *Brucella* immunomodulants in case of simultaneous application with *Cl. perfringens-D* (epsilon) toxoid.

The data explicitly demonstrate a parallel run of the two curves representing the individual results obtained in different immunization trials.

The results displayed in Tables II and III were investigated statistically to define some of the characteristics of the adjuvant activity of immunomodulants prepared from *Brucella* strains. The results of the statistical analysis are shown in Table IV.

Table III

The development of the primary anti-*Cl. perfringens-D* (epsilon) toxin protection and the effect of immunomodulators prepared from different *Brucella* strains

Examination of the reproducibility of results using analysis of variance

Origin of immunomodulators+ (Dose: 0.1 mg)	Relative potency results of the repeated investigations with 95% fiducial limits	
	Investigation I++	Investigation II++
Brucella abortus PH-0048	2.4 (2.1–2.7)	3.2 (2.37–4.32)
Brucella abortus PH-0159	3.2 (2.5–4.1)	4.05 (3.00–5.47)
Brucella abortus PH-0260	1.05 (0.7–1.3)	0.98 (0.72–1.32)
Brucella abortus PH-0371	3.6 (2.7–4.9)	4.85 (3.59–6.55)
Brucella suis PH-0482	2.8 (2.1–3.6)	3.60 (2.66–4.86)
Untreated control	1.0 (0.8–1.3)	1.0 (0.74–1.35)

Analysis of variance: $R^2 = 0.97$ $P = 0.01$ $r^2_{lin} = 0.95$ $P = 0.01$

Source	SQ	DF	MQ	$F_{P=0.01}$
Total	7.6954	5		
Regression	7.4463	2	3.7231	44.83 > 30.82
linear.	7.3181	1		88.13 > 34.12
square	0.1282	1		not significant
Error	0.2491	3	0.083	

+ See footnote to Table I
++ Interval between the two investigations: seven months

Discussion

Discussing the results displayed in Table IV got by computing the data of the repeated active immunization–immunomodulant-treatment trials, we can unanimously conclude that the mechanism of action exerted by two different immunomodulants of *Brucella* origin on the development of the primary antitoxic-type protection, exhibited features of the same type.

The value of the polynomial equation represents:

$$Y = 3.26 - 2.74x + 1.2x^2$$

The regression of the two experiments and the data of the two parallel (repeated) investigations reveal high degree of statistical significance.

Table IV

Analysis of variance of the data computed with the application of polynomial equation and contained in Tables II and III

Analysis of variance (data of Tables II and III are converted to logits)				
Source of variance	SQ	DF+	MQ	$F_{P} = 0.01$
Total	12.8261	9		
Regression	11.6324	2	5.8162	34.1 > 9.5
linear.	10.641	1		62.2 > 12.25
square	1.0282	1		6.0 < 12.25
Error	1.1937	7	0.1705	

Characteristics of the determination-coefficients

Coefficients	Value		P	Per cent share of the total regression
Total-determination-coefficient	R^2	0.91	0.01	—
Linearity-determination-coefficient	$r^2_{lin.}$	0.83	>0.01< 0.02	83.0
Square-determination-coefficient	$r^2_{squ.}$	0.08	not significant	8.0

Polynomial equation: $Y = 3.26 - 2.74x + 1.2x^2$
+DF = Degree of freedom

The two factors defining the regression behave quite differently. The linearity reveals a parallel run of the two curves while no "square" effect can be demonstrated among the factors influencing/defining the action mechanism of the adjuvant activity.

The linearity, according to the values representing the determination-coefficients, takes its share of the activity in 83%, indicating that the data of the repeated investigations performed with both toxoids are practically the same in 80%.

This also shows that the adjuvant activity of *Brucella* immunostimulants exerted on the development of the primary antitoxic-type immunity seems to be a well-defined and applicable phenomenon.

The above data are in full agreement with those achieved during our recently-published experiments concerning the host-defence-increasing activity of immunomodulants of *Brucella* origin (Réthy et al., 1980).

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COMPARISON OF THE LIVER BIOPSY SAMPLE AND THE "WHOLE LIVER" IN RESPECT OF LIPID CONTENT AND FATTY ACID COMPOSITION OF LIPIDS

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Prior to slaughter, samples of 0.08 to 0.15 g were taken from the lobus caudatus of the liver of 9 cows by percutaneous needle biopsy. Two to three hours later the cows were slaughtered and 150–200 g of fresh liver tissue was excised from each of the left, intermediate and right lobe of their livers, and the samples were homogenized. The lipid concentration of the liver biopsy sample and that of the homogenized "whole liver" proved to be nearly identical (45.76 and 45.27 g/kg of wet liver tissue, respectively). Fourteen fatty acids of the lipids were identified by gas chromatography. Of these, 13 showed similar percentages in the lipids of the biopsy sample and in those of the "whole liver". The proportion of myristic acid (C14 : 0), which was present in a relatively low percentage (1.0–2.5%), was significantly higher in the biopsy sample than in the "whole liver". It is suggested that the lipid content and fatty acid composition of the liver biopsy sample are reliable indicators of those of the "whole liver".

Keywords. Lipid content, fatty acid composition, liver biopsy sample, liver disorders, dairy cows.

In dairy cows, disorders of the lipid metabolism accompanied by fatty infiltration of the liver are frequent. Accurate knowledge of the quantity and composition of hepatic lipids is an important contribution to investigations into these disorders. In live animals, hepatic lipids can be determined only in liver biopsy samples.

Liver biopsy is widely used in both the diagnosis of liver diseases of cattle and research work on hepatopathies. The biopsy sample can be used for morphological (histological) and chemical examinations as well. In many cases the two methods are used simultaneously for examination of the liver biopsy specimen (Collins and Reid, 1980). Naturally, the examination of such a specimen can only reveal diffuse hepatopathies. In liver lesions of focal character, the diagnostic value of hepatic puncture performed at random depends on chance, i.e. on the fact that normal or pathologically altered tissue samples are obtained.

The fatty liver syndrome and other hepatopathies of metabolic origin are always diffuse. However, the chance of excising a biopsy specimen truly representing the actual status of the whole liver is open to question. It should

be noted that a liver biopsy sample, which is always excised from the lobus caudatus, weighs 0.1 to 5.0 g depending on the applied biopsy procedure (Ames et al., 1980; Holtenius, 1961; Loosmore and Allcroft, 1951), while the weight of the whole liver is about 5 – 6 kg.

The above question seems to be justified in the light of several reports which indicate that certain, primarily inorganic, substances are not uniformly distributed in the liver. The dry matters of the right and left liver lobes of rats were found to contain different concentrations of Cu (Haywood, 1980). Bingley and Dufty (1972) made similar observations concerning the Cu content of the lobus caudatus and of other lobes of the calf liver.

The possible differences in the concentration of lipid fractions between liver lobes have scarcely been studied. O'Kelly (1974) found that in beef bulls the right and left lobes of the liver contained lower levels of cholesterol and phospholipids and showed higher free cholesterol/total cholesterol ratio than the intermediate lobe.

Our experiments were performed to determine the relationship between the biopsy specimen taken from the caudate lobe of the bovine liver and the homogenized tissue excised in large quantities from different parts of the liver of the same animal, in respect of their total lipid content and fatty acid composition of their lipids. Our aim was to determine the reliability of the liver biopsy technique as an indicator of the whole liver lipid status.

Materials and methods

Nine non-pregnant cows aged 4 – 5 years and sent for slaughter due to different causes (mastitis, leg disorders, infertility) were used. The cows were Hungarian Fleckvieh and Hungarian Fleckvieh x Holstein-Friesian F_1 crosses, and all of them had previously been culled because of agalactia or low milk production (a few litres daily). The cows were transported to the abattoir and subjected to percutaneous needle biopsy after a resting period of 10–12 h, while no feed was available for them. The biopsy was performed in the early morning hours, after infiltration anaesthesia with 5 ml 2% lidocaine hydrochloride, according to the technique described by Horváth et al. (1967). After removing non-parenchymatous tissues, biopsy samples weighed 0.08–0.15 g.

The cows were slaughtered 3–4 h after sampling. Immediately after slaughter, fresh liver samples were excised from several sampling sites of the left, intermediate and right liver lobes, amounting to a total sample weight of 150–200 g per lobe, i.e. 450–600 g per liver. The liver samples were homogenized in a BIOMIX laboratory homogenizer and freeze-stored at –25 °C until used for further analysis. The samples taken after slaughter are hereinafter referred to as "whole liver" samples.

Total lipid content of the biopsy samples and of the "whole liver" samples taken after slaughter was determined by the method of Folch et al. (1957). The 0.08–0.15 g biopsy samples were processed in their entirety, while of the "whole liver" samples 4 g quantities were used for processing.

Fatty acid composition of lipids extracted from liver biopsy and "whole liver" samples was determined by gas-chromatographic analysis. Fatty acids of lipids extracted in chloroform-methanol were evaporated and subsequently transformed to methylesters in the presence of sulphuric acid as catalyst (Chalvardjian, 1964).

Fatty acid–methylesters were analysed by gas chromatography in a CHROM-42 apparatus as described earlier (Husvéth et al., 1982). The quantitative determination of fatty acids was based on measurement of the areas beneath the peaks representing the different components, and the results were expressed in percentage of the total fatty acid content.

On the whole, the following 14 fatty acids were identified: C 12 : 0, C 14 : 0, C 15 : 0, C 16 : 0, C 16 : 1, C 17 : 0, C 18 : 0, C 18 : 1, C 18 : 2, C 18 : 3, C 20 : 0, C 20 : 4, C 20 : 5, C 22 : 0. The other fatty acids, present in lower quantities, were not identified; these are mentioned as "other fatty acids".

Statistical evaluation of the results was performed by using Student's paired t test.

Results

The lipid content of the liver biopsy samples and of the "whole liver" samples is shown in Table I. Biopsy samples and "whole liver" specimens gave practically identical results in respect of their lipid content. Total lipid values obtained by us (45.76 and 45.27 g/kg of wet liver tissue) correspond to average values obtained for the liver of nonlactating or low-producing cows.

The fatty acid composition of hepatic lipids is shown in Table II.

Table I

Comparison of the biopsy sample and the "whole liver" in respect of lipid content in cows

	Lipid content	
	Biopsy sample	"Whole liver"
Mean value, g/kg wet liver tissue	45.76	45.27
Standard error of mean (SEM)	2.38	0.92
Coefficient of variation, %	15.6	6.1
Number of samples	9	9

Table II

Comparison of the biopsy sample and the "whole liver" in respect of fatty acid composition of their lipids

Fatty acid		Percentage of fatty acid ± standard error of mean (SEM)		Significance of difference
Name	Designation	Biopsy sample	"Whole liver"	
Lauric acid	C 12 : 0	0.21 ± 0.02	0.20 ± 0.02	N. S.
Myristic acid	C 14 : 0	2.45 ± 0.16	1.03 ± 0.09	P < 0.001
Pentadecanoic acid	C 15 : 0	0.46 ± 0.04	0.53 ± 0.03	N. S.
Palmitic acid	C 16 : 0	15.86 ± 1.29	16.23 ± 1.06	N. S.
Palmitoleic acid	C 16 : 1	1.73 ± 0.14	1.82 ± 0.12	N. S.
Margaric acid	C 17 : 0	1.08 ± 0.09	1.11 ± 0.07	N. S.
Stearic acid	C 18 : 0	30.80 ± 0.65	31.00 ± 0.63	N. S.
Oleic acid	C 18 : 1	14.50 ± 1.10	15.20 ± 0.83	N. S.
Linoleic acid	C 18 : 2	10.77 ± 1.03	11.13 ± 1.05	N. S.
Linolenic acid	C 18 : 3	0.95 ± 0.11	0.94 ± 0.13	N. S.
Arachidic acid	C 20 : 0	0.37 ± 0.05	0.36 ± 0.05	N. S.
Arachidonic acid	C 20 : 4	8.77 ± 0.49	8.72 ± 0.45	N. S.
Eicosapentaenoic acid	C 20 : 5	0.76 ± 0.17	0.68 ± 0.15	N. S.
Behenic acid	C 22 : 0	6.71 ± 0.74	6.89 ± 0.74	N. S.
'Other fatty acids"		4.58 ± 0.54	4.16 ± 0.36	N. S.

Of the 14 identified fatty acids, 13 showed no significant difference ($P > 0.05$) in respect of their proportion in lipids of the liver biopsy sample and the "whole liver" specimen. Similar observations were made on the tota proportion of the "other fatty acids". The only significant difference between fatty acids of the biopsy sample and of the "whole liver" was in the proportion of myristic acid. Although both types of samples contained myristic acid in a relatively low percentage (1.0–2.5%), liver biopsy samples contained significantly ($P < 0.001$) higher amounts of it than "whole liver" samples.

Discussion

According to the present results, the lipid content of the liver biopsy sample and of the "whole liver" proved to be practically identical, suggesting that the lipid content of the liver biopsy sample was a reliable indicator of that of the "whole liver". In metabolic disorders and toxicosis the liver undergoes diffuse fatty degeneration. Therefore, in these cases the organ can be considered homogeneous, in the same way as the healthy livers examined by us.

In the examination of homogeneous liver tissue, excision of connective-tissue septa and small biliary vessels together with the biopsy sample may constitute a source of error. This primarily occurs if the biopsy is performed "at random". However, tissues of nonparenchymatous origin can easily be removed. In doubtful cases, the chemical analysis must be accompanied by a simultaneous histological examination. Only sampling from laparotomized animals is reliable, which can be controlled through the wound of the abdominal wall (Ames et al., 1980). This procedure is, however, lengthy, laboursome and entails a considerable risk of infection.

Analysis of the fatty acid composition of hepatic lipids revealed that only myristic acid (C 14 : 0) was present in a significantly different proportion in the biopsy sample than in the "whole liver". At present, no satisfactory explanation is available for this phenomenon. Since myristic acid is a relatively short-chain and rapidly metabolizing fatty acid, possibly the few hours elapsed between biopsy and slaughter may have accounted for its partial disappearance. All other fatty acids (among them the predominant C 16 : 0, C 18 : 0, C 18 : 1 and C 18 : 2) showed practically identical proportion in the liver biopsy sample and in the "whole liver". Thus, the biopsy sample was a reliable indicator of the whole liver also in respect of the fatty acid composition of lipids. In a previous work (Husvéth et al., 1982) we found similar proportions of C 16 : 1 and C 18 : 0 in liver biopsy samples of dairy cows in the peripartal period as in the present experiment. In the peripartal period, the percentage of fatty acids C 14 : 0, C 18 : 2 and C 20 : 4 was two-third, half and one-third, respectively, of the quantities determined in the present work. On the other hand, in the peripartal period C 16 : 0 and C 18 : 1 showed proportions approximately one-fifth higher than those found in the present case. The observed differences are due to the different reproductive status of the cows used in the two experiments.

In summary, we can state that the liver biopsy sample is a reliable indicator of the homogeneous "whole liver" in respect of lipid content and fatty acid composition. The status of the healthy liver and of that undergone diffuse changes can be followed closely by chemical analysis of the biopsy sample.

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CHANGES IN THE PLASMA CONCENTRATION OF SEXUAL STEROIDS IN DOMESTIC HENS DURING FORCED AND HORMONALLY-INDUCED MOLT

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Changes in the function of the ovary were studied in two groups of hens subjected to experimental molt. In the first group, forced molt was induced by biotechnical methods (withdrawal of feed, drinking-water and light, and decrease of temperature). In the other group, molt was induced by hormonal treatment (progesterone and triiodothyronine (T_3)). The change of feathers was of different character in the two groups.

The concentration of progesterone in the blood plasma increased significantly in the period of feather loss, while other sexual steroids (testosterone, 17-β-oestradiol) were at low levels, and oestrone even decreased significantly.

In later stages of molting, at the time of new feather outgrowth, progesterone concentration tended to decrease. This was accompanied by a significant rise in the levels of oestrone and testosterone. The level of 17-β-oestradiol was low also in this period.

The authors suggest that the increase of plasma progesterone at the beginning of molting may be one of the factors responsible for inducing the process. In this period, the low level of 17-β-oestradiol and the significant decrease of oestrone concentration may have a permissive effect on progesterone, thus facilitating the initiation of molting.

Keywords. Molting, sexual steroids, progesterone treatment, triiodothyronine treatment.

In the domestic hen, the number of eggs laid during the oviposition cycle is fundamentally influenced by the time and intensity of molting. This characteristic physiological process of birds has been studied extensively for a long time by various experimental approaches. Initially, the primary responsibility of the thyroid gland was suggested (Cole and Hutt, 1928). Later on, the primary involvement of other endocrine organs, such as the pituitary gland (Juhn and Harris, 1958), the adrenals (Brake et al., 1979; Perek and Eckstein, 1959), or the gonads (Harris and Shaffner, 1957; Juhn and Harris, 1956, 1958; Tanabe et al., 1956) was stressed.

Presumably, the complicated mechanism of molting results from an interrelationship of several hormones, and not from the function of a single endocrine organ. In their investigations into the influence of thyroid-adrenal interaction, Brake et al. (1979) failed to detect an unequivocal relationship.

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In the light of the literature available so far, a characteristic interaction between the thyroid gland and gonads seems to represent the underlying mechanism of molting (Farner and King, 1972). The functional interaction of these two endocrine organs and its association with the process of molting have been studied by our research team in several avian species (Péczely and Pethes, 1980, 1982; Pethes et al., 1982; Szelényi et al., 1980, 1981).

The present experiments were aimed at studying the changes of plasma concentrations of sexual steroids in different stages of molting in domestic hens subjected to forced molt by biotechnical methods and by hormonal treatment.

Materials and methods

In the first experiment, forced molt was induced in 63 weeks old Hybro parent hens by withdrawal of feed, drinking-water and light, and by decreasing the ambient temperature. The technology of forced molt was described previously (Pethes et al., 1982; Szelényi et al., 1981). Blood samples were collected weekly from a representative group of 7 birds on each occasion. The first blood sampling was performed at the beginning of the experiment. The second (day 7) and third (day 14) blood samples were withdrawn after the loss of contour feathers and down feathers. By day 21, the down feathers had grown out again in the cervical and thoracic region. and of the contour feathers the pinions had reappeared. By the last blood-sampling (day 35) all birds had acquired a complete new feathering.

Egg production of the hens was 37% at the beginning of the experiment. In forced molt, the layers ceased to produce eggs within 1—2 days. In the oviposition cycle following molt, production reached 50% in the 13th week.

The second experimental group included 7 hens selected from a 49 weeks old Hybro parent stock kept on deep litter. The experimental birds were kept in an enclosed area of 8 m^2 in the layer house, thus they were exposed to the same environmental factors as the whole laying stock of 4800 hens. The lighting programme consisted of a light period of 18 h and a dark period of 6 h. Ambient temperature was 12–16 °C in the layer house during the experiment. Drinking-water was given ad libitum. Laying hens were given a daily ration of 0.152 kg layer feed + 0.010 kg barley; the experimental group received the same feeding.

Egg production of the laying stock was 57% at the beginning of the experiment. The randomly-selected layers ceased to lay eggs on the second day of hormonal treatment.

The experimental birds were given 5 mg progesterone (Glanducorpin oil-emulsion injection, G. Richter Pharmaceutical Works, Budapest) i.m. and 0.02 mg liothyroninum hydrochloricum (Liothyronine tablets, G. Richter

Pharmaceutical Works, Budapest) per os daily, for 21 days. Molting began on the 10th day of treatment, and by the 35th day the birds had acquired a complete new feathering. Hormonally-induced molt had a less drastic effect than forced molt. The stages of feather exchange (feather loss, and outgrowth of new feathering) were not demarcated so sharply, and the process was more similar to natural molting than forced molt. On the 49th day of the experiment, 50% of the treated stock had already come into lay. Blood samples were taken weekly from the experimental birds.

All blood samples were centrifuged at 3000 rpm and the plasma was stored at −20 °C until used. Plasma samples were fractionated by chromatography on a Sephadex LH-20 column and assayed for the following sexual steroids by simultaneous radioimmune analysis (Péczely et al., 1980): progesterone (PROG), testosterone (TEST), oestrone (E_1) and 17-β-oestradiol (E_2).

Results

Experimental group No. 1 (forced molt). Soon after the beginning of the experiment, the plasma level of PROG increased significantly, followed by a gradual decrease. The concentration decrease found at the end of the experiment was significant.

The plasma levels of TEST were high in the last two groups (at the time of new feather outgrowth), and the rise was significant as compared to the values found on day 14.

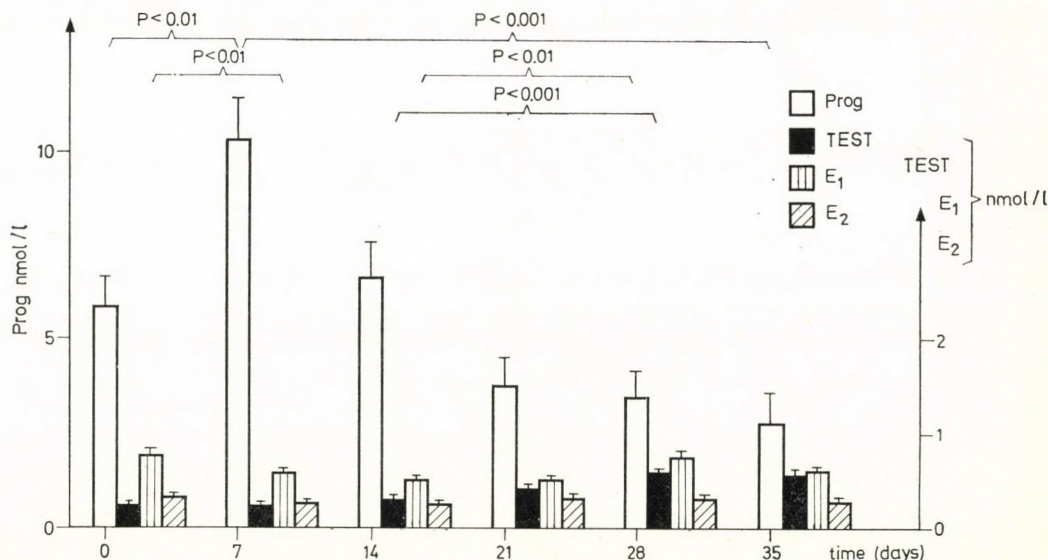

Fig. 1. Changes in plasma levels of sexual steroids in the forced-molt group ($n = 7$)

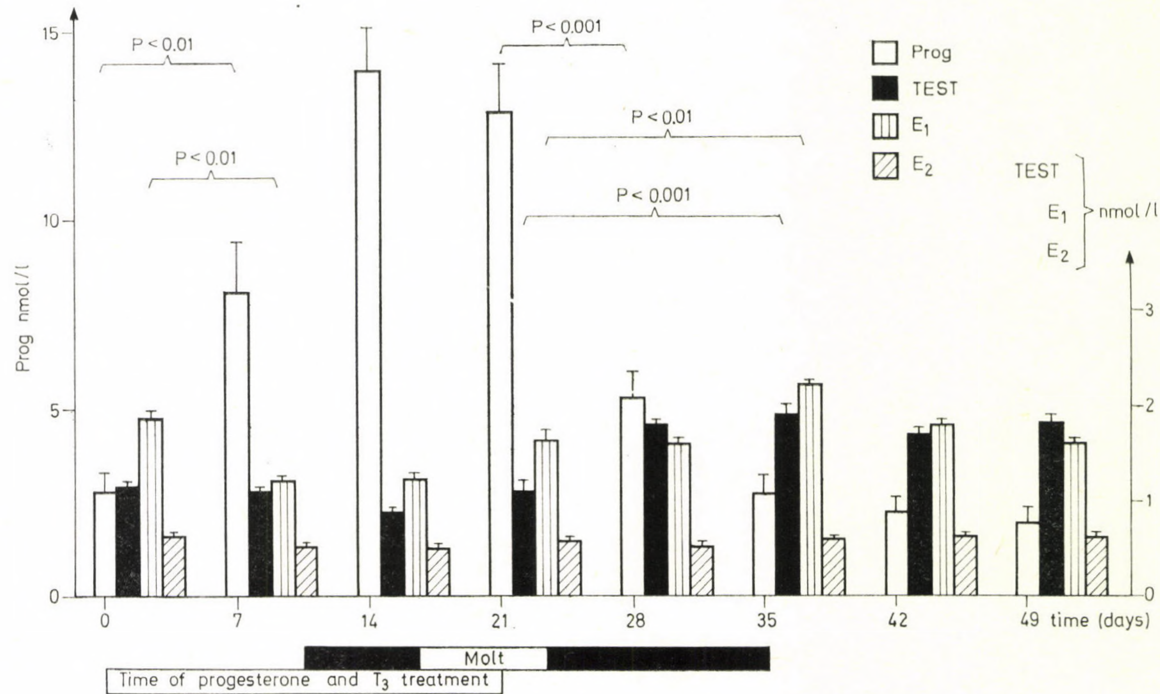

Fig. 2. **Changes in plasma levels of sexual steroids in the group molted by hormonal treatment ($n = 7$)**

Of the oestrogens, E_2 was at a low level throughout, while the E_1 levels showed a significant decrease followed by an increase parallel with that of TEST from day 14 onwards (Fig. 1).

Experimental group No. 2 (hormonally-induced molt). Treatment induced a marked rise in the plasma level of PROG, which remained high until a significant drop in a late phase of the experiment. Plasma concentration of TEST increased in the last third of treatment, in the period of new feather outgrowth (from days 28–35 onward). In this period, E_1 showed a similar increase, which followed the significant decrease of the E_1 concentration observed at the beginning of the experiment. E_2 was at a low level throughout the experiment (Fig. 2).

Discussion

The important role of PROG in molting is indicated by data of the literature on successful induction of molting with this hormone (Adams, 1956; Harris and Shaffner, 1957; Juhn and Harris, 1956). In our earlier investigations (Pethes et al., 1982; Szelényi et al., 1981), we also observed a rise in the plasma concentration of PROG at the beginning of molting, in the period of feather loss. Apart from the above authors, only Furr (1973) measured the plasma levels of PROG in molting hens, and found low PROG concentrations in these birds. However, since this latter investigation was not extended to the whole process of molting, it cannot be excluded that the determination of PROG was performed in the period when the PROG level was already low.

In both experimental groups of the present study, a significant rise of plasma PROG levels occurred in the period of feather loss. This rise was not followed by ovulation, and oviposition was also completely absent. This is remarkable since in laying hens the plasma concentration of PROG is known to reach a peak 4 to 7 h before ovulation, otherwise it is characterized by low base values (Furr et al., 1973; Laquë et al., 1975; Péczely et al., 1980; Shababi et al., 1975). The birds of our control groups showed these base levels. Subsequently, the plasma level of PROG increased, remained consistently high and did not show an abrupt preovulatory peak. This was responsible for the complete absence of ovulation and oviposition. The consistently high PROG level can be attributed partly to hormonal interactions elicited by the continuous influence of environmental factors ("stress"), partly to the slow absorption of the oil-emulsion PROG preparation given intramuscularly.

On the basis of the present experiments, of our earlier results (Pethes et al., 1982; Szelényi et al., 1981) and data of the literature (Adams, 1956; Harris and Schaffner, 1957; Juhn and Harris, 1956), the increase of PROG concentration occurring at the beginning of molting may be one of the factors

responsible for inducing the process of molting. Presumably, PROG is one of the factors which, acting directly on the papillae, cause deplumation.

In our study, sexual steroids other than PROG showed low plasma concentrations in the period of feather loss. Of these, the low concentration of E_1 developed after a significant drop. This is in accordance with the molt-inhibiting effect of exogenous oestrogens that has been presumed for a long time (Onishi et al., 1955; Tanabe and Katsuragi, 1962). The above finding also agrees well with the results of Juhn and Harris (1956), who reported that testosterone propionate induced molting only with a considerable delay. They suggested that in this case molting was not due to a direct stimulatory effect of TEST, but to a later reaction.

E_2 was at a low base level throughout the experiments. On the other hand, the concentration of E_1 and TEST increased significantly in the period of new feather outgrowth. We assume that these higher hormone levels act upon the outgrowth of new feathering and "prepare" certain organs (oviduct, medullary bone) of laying hens for physiological processes associated with egg-laying. In laying hens, Petterson and Common (1972) detected regular, while others (Laquë et al., 1975; Péczely et al., 1980; Senior, 1974) irregular oestrogen peaks. The role of these peaks has not been elucidated yet, but it is presumed that they are not primarily related to ovulation.

As opposed to other authors, Etches and Cunningham (1977) suggested that in laying hens TEST plays a primary role in triggering the preovulatory LH peak. However, in our experimental groups the significant rise in the plasma concentration of TEST was not followed by ovulation and a new laying cycle.

These observations are in accordance with results of other authors (Péczely et al., 1980; Shababi et al., 1975), who reported irregular peaks in the plasma concentration of TEST during the ovulation cycle of the laying hen. These results query the primary role of this hormone in the regulation of ovulation.

In conclusion, the significant drop of E_1 level at the beginning of the experiment (at the time of feather loss) must also be considered important. The low levels of these sexual steroids have a permissive influence on the increased PROG concentration, thus rendering possible the initiation of molting.

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COLD-EXPOSURE-ELICITED MODIFICATIONS IN THE THYMUS, BURSA OF FABRICIUS, ADRENALS AND BLOOD OF CHICKENS

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Studler-Cornish chickens aged 1, 4, 12 and 30 days were maintained at 4 °C during 40 min, with a "warm" interval of 10 min, and killed either immediately thereafter or after two hours. Weight of the thymus and bursa of Fabricius, and their content of glycogen and free amino nitrogen, adrenal content of ascorbic acid and glycogen, and serum cholesterol concentration failed to show a clear stress state, though some involution phenomena were found in the bursa of 4-day-old chickens. Modifications are age-dependent, especially in 30-day-old birds being highly different from those at other ages. A cold action via the thyroid rather than the adrenal cortex is supposed in young chickens.

Keywords. Cold exposure, chicken, changes in thymus, bursa of Fabricius, adrenals, blood, age-dependency.

The effects of cold upon the avian organism have been investigated widely. The glycogen utilization in the skeletal musculature is enhanced (Lawrie, 1966; Parker and George, 1974, 1975), immunobiological processes are depressed (Rao and Glick, 1977; Thaxton, 1978), and enzyme activities and tissue composition are considerably modified in cold (David and Zolman, 1971; Giurgea and Manciulea, 1975; Lawrie, 1966; Manciulea et al., 1976; Moss and Balnave, 1978).

Having in mind the practical importance of such studies for intensive avian rearing, we investigated some reactions of the chicken's organism to cold exposure during the first month after hatching.

Materials and methods

Experiments were done on tetralinear hybrid Studler—Cornish chickens, aged 1, 4, 12 and 30 days. Three groups, each consisting of 8 chickens, were formed for each age. Group A: control group, kept at 22 °C throughout; group B: kept for 20 min at 4 °C, then for 10 min at 22 °C and for another 20 min at 4 °C. These chickens were killed immediately after the second exposure to cold. Group C was kept as group B, but the chickens were killed two hours after the second cold exposure. The rearing conditions were the same as in avian farms. The chickens were fed with standard, age-fitted concentrated

fodder. Water was given ad libitum. Food was withdrawn during the last 18 h before killing. The animals were killed by decapitation. The organs were quickly excised and weighed on a torsion balance. Glycogen content was determined (Montgomery, 1957) in the thymus, the bursa of Fabricius (in the following, bursa) and the right adrenal; free amino-acid nitrogen (Rác, 1959) in the thymus and bursa, and ascorbic acid (Klimov, 1957) in the left adrenal. Cholesterol was determined (Zak, 1954) in the serum.

Mean values for each age, group, organ and parameter were checked for homogeneity by Chauvenet's criterion, aberrant individual values were deleted. Student's t test was used for comparison and P = 0.05 was considered the threshold for statistical significance. Differences of B and C values against A were calculated, and expressed in per cent.

Table I

Control values in thymus, bursa of Fabricius, adrenals, and blood serum of chickens

Age (days)	1	4	12	30
		Bursa of Fabricius		
Glycogen (mg/g)	1.64 ± 0.13	0.86 ± 0.10	0.40 ± 0.11	0.48 ± 0.06
Free amino acids (mg/100 g)	27.95 ± 5.23	41.40 ± 10.70	19.40 ± 4.48	0.56 ± 0.12
Weight (mg)	51.42 ± 0.16	53.68 ± 4.19	77.93 ± 17.98	220.93 ± 26.48
		Thymus		
Glycogen (mg/g)	1.07 ± 0.12	0.81 ± 0.09	1.03 ± 0.21	0.77 ± 0.10
Free amino acids (mg/100 g)	12.74 ± 2.89	25.37 ± 5.78	18.71 ± 1.52	0.91 ± 0.13
Weight (mg)	121.12 ± 12.32	110.82 ± 10.08	151.81 ± 23.30	202.68 ± 21.64
		Adrenals		
Glycogen (mg/g)	4.42 ± 0.53	2.27 ± 0.32	0.97 ± 0.04	0.51 ± 0.07
Ascorbic acid (μg/gland)	17.20 ± 1.81	22.42 ± 0.85	13.90 ± 0.90	29.90 ± 1.61
		Blood serum		
Cholesterol (mg/100 ml)	635.14 ± 21.18	592.71 ± 22.30	235.12 ± 25.83	199.50 ± 12.13

Results

The means $\pm$ standard errors for the thymus, bursa, adrenals and blood serum of control chickens are presented in Table I. The number of individual values was 7 or 8.

Modifications in the thymus and bursa are given in Figs 1 and 2.

Thymus weight increased in cold-exposed 1-day-old chickens by 44% in group C; bursa weight at the same age by 22% in group B. A weight decrease was observed in the bursa of 4-day-old chicks (−21% in group C). The other differences are not significant. Glycogen concentration was increased in the thymus of 4-day-old chicks (group B and C), and of 12-day-old ones (group

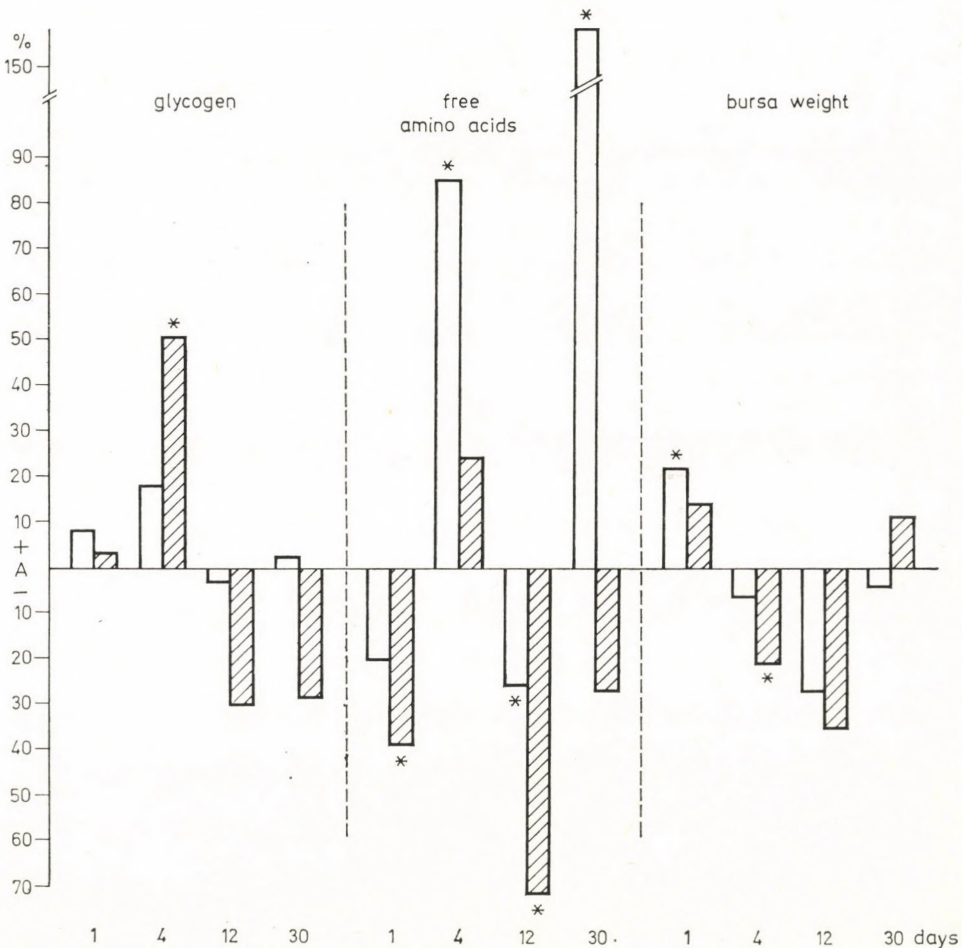

Fig. 1. Modifications in the bursa of Fabricius of cold-exposed chickens (percentage differences vs. control values). White columns: group B; striated columns: group C; *: statistically significant differences, $P < 0.05$

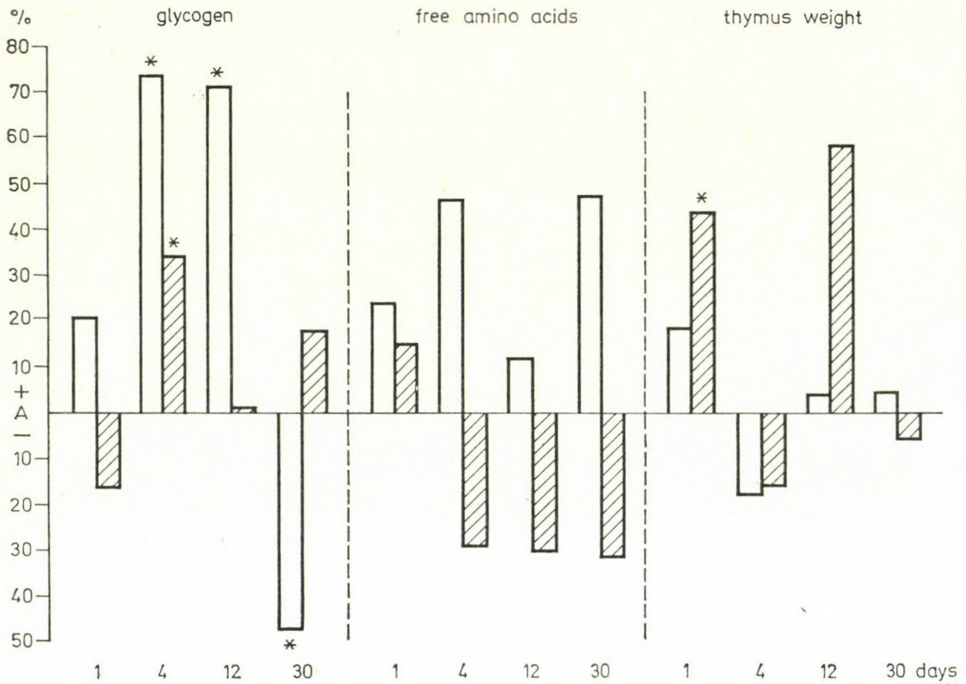

Fig. 2. Modifications in the thymus of cold-exposed chickens (percentage differences vs. control values). White columns: group B, striated columns: group C; *: statistically significant differences, $P < 0.05$

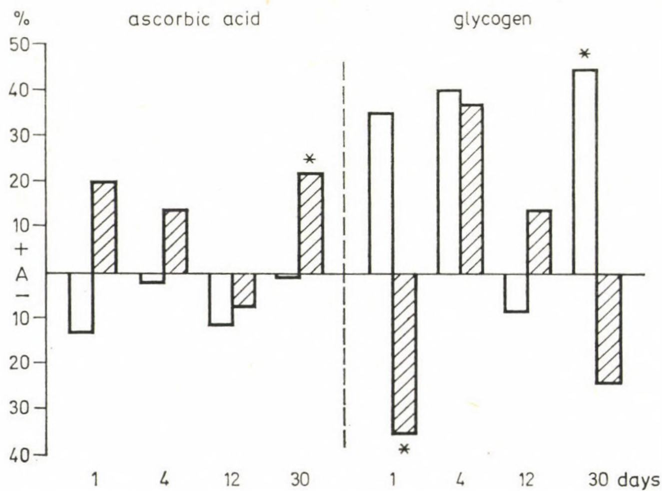

Fig. 3. Modification in the adrenals of cold-exposed chickens (percentage differences vs. control values). White columns: group B; striated columns: group C; *: statistically significant differences, $P < 0.05$

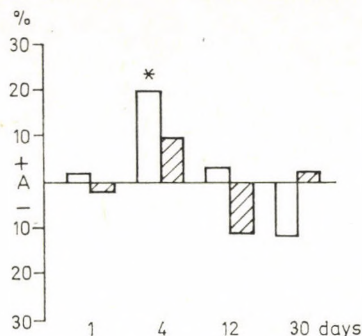

Fig. 4. Modifications in the serum cholesterol concentration of cold-exposed chickens (percentage differences vs. control values). White columns: group B; striated columns: group C; *: statistically significant differences, $P < 0.05$

B only); a significant decrease was noticed for 30-day animals of the same group. The unique significant change in the glycogen concentration of the bursa was an increase in group C of 4-day chickens. Free amino-acid nitrogen concentration showed no significant change for the thymus. In the bursa, large decreases occurred in the one- and 12-day groups, and great increases in the four- and 30-day ones.

As shown in Fig. 3, the only significant modification of the adrenal ascorbic acid concentration was a 22% increase in the 30-day chickens of group C. The glycogen concentration showed a 35% decrease in the one-day chickens of group C and a 45% increase in the 30-day chickens of group B.

Blood serum cholesterol concentration (Fig. 4) was not significantly modified. Only in 4-day chickens of group B, a 20% increase occurred.

Discussion

Our results show that there are differences in the reaction to cold, on the one hand between both lymphoid organs, on the other hand between chickens of different ages. The weight of both the thymus and the bursa increased in 1-day chickens; however, while the modification is very fast in the case of the latter (20% increase in less than an hour), the reaction of the former became significant only in the period of recovery (i.e., in group C). In older chickens, no significant weight increase occurred in either organ.

Some of the changes might suggest a state of stress (Pora et al., 1968; Toma and Giurgea, 1974): the increase of glycogen concentration of the thymus in 4- and 12-day chickens (group B), and the loss of weight of the bursa in 4-day ones (group C). Nevertheless, as there was no decrease in the ascorbic acid concentration in the adrenals, it is possible that no stress occurred and

that the changes in the lymph organs were not mediated by a glucocorticosteroid hypersecretion.

If the changes observed in the lymph organs are not manifestations of a stress, the mechanism by which cold affects the thymus and the bursa deserves interest. A possible pathway is that assumed by Rao and Glick (1977): hypothalamus–hypophysis–thyroid. In our opinion, the involvement of the thyroid is plausible, as this organ plays a role in stress events (Kuzmak, 1982). Thyroid is already functioning in the embryo (Hansborough and Kham, 1951) and its participation in thermoregulation has been shown in newly-hatched chickens (Freeman, 1971), meanwhile the hypothalamus–hypophysis–adrenal axis does not reach maturation until the age of three weeks (Avrutina and Kisljuk, 1978).

Another possible pathway might be led by the adrenal medulla. The catecholamine concentration of the blood rises under the action of the cold (Jorenen et al., 1976). Literature contains various views concerning the mechanism by which cold exposure activates the adrenal medulla, via the thyroid, or via the sympathetic nervous system (David and Zolman, 1971; Freeman, 1971; Lawrie, 1966).

As a third possibility, the adrenal cortex might be involved, even if, in so young chickens, the mechanism is not a typical stress. A support for this view might be the increase of the ascorbic acid level in the adrenals during the recovery from the cold aggression (group C); this increase, occurring at the majority of the ages investigated, might be interpreted as an "overshoot" after a previous decrease, which was not noticed in our experimental schedule.

Of course, our results can but underline the possible complexity of the pathways and mechanism of action of the cold exposure in chickens, as well as their pronounced age-dependence. On the other hand, modifications like those we noticed in the thymus and the bursa might substantiate the existing data on alterations in the immunological capacity of cold-exposed young chickens (Thaxton, 1978).

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EFFECTS OF TYPE AND INTENSITY OF ASSISTANCE ON ACID-BASE BALANCE OF NEWBORN CALVES

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The acid–base balance of 106 newborn calves removed by traction or Caesarean section in anterior or posterior presentation from primi- or multiparous cows was examined. Calves in anterior presentation born by traction were divided into two subgroups: extracted by moderate or intensive assistance.

Besides disturbances of diaplacental circulation it was the way of assistance which influenced the acid–base balance of newborn calves during the first 10 min of life. In the case of traction it is the duration of the passage through the birth canal and, within this period, the duration of the compression of the umbilical cord which may play a decisive role.

Keywords. Type and intensity of assistance, acid–base balance, newborn calves.

At birth mammals are exposed to hypoxia and to the acidosis resulting from the utero-placental circulation being disturbed by rupture of membranes and contraction of the uterus. The degree of hypoxia and acidosis depends on the length of the period between the complete separation from the dam's blood circulation and the start of spontaneous respiration, and on the degree of disturbance of diaplacental gas exchange during calving (Walser and Maurer–Schweizer, 1978). Even in case of normal calving, all newborn calves suffer from a slight, combined respiratory and metabolic acidosis, which develops gradually during calving; the pH does not drop below 7.2 and its postnatal compensation is fast (Ammann et al. 1974; Bodenberger, 1979; Eichler–Steinhauff, 1977; Maurer–Schweizer et al., 1977*a*; Moore, 1969; Schlerka et al., 1979; Szenci et al., 1982).

In the present study we attempted to answer the following questions: to what extent does the type of obstetrical assistance (traction or Caesarean section) influence the acid–base balance of newborn calves, born of primi- or multiparous cows with anterior or posterior presentation.

Materials and methods

One hundred and six complicated calvings of black-and-white and red--and-white Dutch Friesian cows were studied. Three of the calves were premature. Traction was applied within 3–4 h, while a Caesarean section was

performed within 4–5 h following rupture of membranes. Traction was applied by three persons, always under guidance of a veterinarian. The group of calves with an anterior presentation and extracted by traction was divided into two subgroups: traction with moderate, or intensive assistance. The latter was required — in most cases — by the hip lock flexion of the calves.

The newborn calves weighed 38–52 kg. Calves delivered by Caesarean section were generally heavier than those delivered by traction ($P < 0.001$) and in the case of Caesarean section the calves born of cows weighed more than those born of heifers ($P < 0.05$).

Blood samples were taken from the jugular vein of the calves immediately after birth and 10 min later, under anaerobic conditions in heparinized syringes. Immediately thereafter the syringes were capped, and stored in ice until used. The blood pH, pCO_2, and pO_2 were determined within one hour after sampling with a blood-gas analyser, ABL 1, Radiometer, Copenhagen, at 37 °C temperature. The results were then corrected to 39 °C, the average body temperature of calves. Haemoglobin was measured by the cyanmethaemoglobin method in a Vitatron haemoglobinometer.

Other acid–base parameters (BE: base excess, EBE: extracellular base excess, act. HCO_3^-: actual hydrogen carbonate) were calculated as described by Szenci and Nyírő (1981).

Statistical analysis was performed by Student's *t* test.

Results

Immediately after delivery by traction with an anterior presentation, the pH values of the calves born of primiparous cows were lower ($P < 0.05$) than those born of multiparous ones (Table I). Ten minutes later, differences were still detectable.

In the case of traction, pCO_2 values of calves born of heifers were higher than those born of multiparous cows, but the differences were not significant. Ten minutes later, the differences between those groups disappeared. The pO_2 values changed similarly. Immediately after birth, the metabolic parameters (BE, EBE, act. HCO_3^-) in both types of intervention were lower in calves born of heifers than in those born of multiparous cows but the differences were not significant. Ten minutes after birth, the metabolic values of the two groups were significantly different. No differences were found between calves born of heifers and those born of multiparous cows after Caesarean section, but the former had lower values in both blood samples.

Immediately after birth, there were no significant differences between the pH values of newborn calves with an anterior presentation and delivered by traction or Caesarean section, but after traction heifer-calves and cow-

Table I

The acid–base balance of newborn calves of primi- and multiparous cows after an assisted birth (anterior presentation)

Parameters	Blood levels immediately post partum		Blood levels 10 min post partum	
	heifer	cow	heifer	cow
	Traction			
	n = 18	n = 28	n = 18	n = 24
pH	7.098 ± 0.103	7.162 ± 0.089*	7.054 ± 0.121	7.131 ± 0.087*
pCO_2 (kPa)	10.2 ± 1.6	9.4 ± 1.3	9.5 ± 1.1	9.4 ± 1.3
pO_2 (kPa)	4.3 ± 0.8	3.9 ± 0.8	4.5 ± 0.4	4.5 ± 0.7
BE (mmol/l)	−6.3 ± 4.5	−3.8 ± 4.0	−9.6 ± 5.5	−5.9 ± 4.2*
EBE (mmol/l)	−4.4 ± 4.2	−2.3 ± 3.6	−8.0 ± 5.2	−4.3 ± 4.0*
act. HCO_3^- (mmol/l)	23.0 ± 3.5	24.4 ± 2.9	19.6 ± 4.0	22.6 ± 3.4*
	Caesarean section			
	n = 29	n = 14	n = 29	n = 14
pH	7.162 ± 0.148	7.172 ± 0.118	7.132 ± 0.147	7.169 ± 0.116
pCO_2 (kPa)	8.9 ± 1.3**^{T}	9.1 ± 1.2	8.9 ± 0.9*^{T}	8.6 ± 0.9*^{T}
pO_2 (kPa)	3.3 ± 0.8***^{T}	3.7 ± 0.3	4.4 ± 0.7	4.4 ± 0.5
BE (mmol/l)	−4.3 ± 6.9	−3.9 ± 5.6	−6.0 ± 7.0	−5.0 ± 6.2
EBE (mmol/l)	−2.9 ± 6.4	−2.3 ± 5.1	−4.3 ± 6.8	−3.6 ± 5.8
act. HCO_3^- (mmol/l)	23.7 ± 5.1	24.4 ± 4.1	22.2 ± 5.1	23.0 ± 4.8

*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$
T (traction): Caesarean section

Table II

The acid–base balance of newborn calves with anterior presentation extracted by moderate or intensive assistance

Parameters	Blood levels immediately post partum		Blood levels 10 min post partum	
	Moderate assistance	Intensive assistance	Moderate assistance	Intensive assistance
	n = 27	n = 16	n = 24	n = 17
pH	7.172 ± 0.081	7.077 ± 0.069***	7.165 ± 0.062	6.991 ± 0.071***
pCO_2 (kPa)	9.8 ± 1.5	9.7 ± 1.2	9.2 ± 0.8	10.4 ± 1.3**
pO_2 (kPa)	3.7 ± 0.6	4.8 ± 0.8***	4.6 ± 0.5	4.3 ± 0.6
BE (mmol/l)	−2.6 ± 2.5	−8.6 ± 3.1***	−4.4 ± 2.8	−11.8 ± 3.6***
EBE (mmol/l)	−0.7 ± 2.3	−7.5 ± 3.3***	−3.3 ± 2.4	−9.9 ± 3.6***
act. HCO_3^- (mmol/l)	25.9 ± 1.5	20.7 ± 2.6***	23.7 ± 2.0	18.5 ± 3.3***

**$P < 0.01$
***$P < 0.001$

calves had lower values. In both blood samples pCO_2 values were lower after Caesarean section than after traction. The calves born of cows had almost the same values in both groups.

The group of calves with an anterior presentation delivered by traction was divided into two subgroups: traction with moderate or intensive assistance (Table II). Immediately after birth, the pH- and metabolic values of calves extracted by intensive assistance were significantly lower than in calves extracted by moderate assistance. The values of pCO_2 showed no difference while pO_2 values were higher ($P < 0.001$).

Ten minutes after calving, significant inter-group differences between the pH values and metabolic parameters could still be detected. In calves extracted by intensive assistance, the pCO_2 values were increased. The significant differences between the values of pO_2 had disappeared.

Immediately and 10 min after calving, pH as well as pO_2 values and metabolic variables were lower in posterior presentation after traction than after Caesarean section, but the differences were not significant (Table III). In both blood samples, pCO_2 levels were lower after Caesarean section than after traction, but significant differences were only found after 10 min.

Examining the values immediately after calving we found that in the case of posterior presentation and traction, pH values laid between the values of the anteriorly-presented calves born of heifers and those born of cows, whereas in the case of Caesarean section, pH values of this group were lowest. The values of pO_2 immediately after birth were significantly lower ($P < 0.01$) after Caesarean section in anterior than in posterior presentation. After 10 min, no differences were found. The other parameters showed up changes very similar to that of the pH.

Table III

The acid–base balance of newborn calves with posterior presentation delivered by traction or Caesarean section

Parameters	Blood levels immediately post partum		Blood levels 10 min post partum	
	Traction	Caesarean section	Traction	Caesarean section
n	6	8	6	8
pH	7.126 ± 0.114	7.148 ± 0.124	7.058 ± 0.112	7.133 ± 0.122
pCO_2 (kPa)	9.9 ± 1.5	9.3 ± 1.4	10.4 ± 0.4	9.2 ± 1.0*
pO_2 (kPa)	4.1 ± 1.3	4.3 ± 0.6	4.1 ± 0.8	4.6 ± 0.5
BE (mmol/l)	−5.4 ± 4.5	−4.7 ± 6.1	−8.2 ± 6.0	−5.4 ± 6.4
EBE (mmol/l)	−3.7 ± 3.7	−3.3 ± 5.9	−6.5 ± 5.4	−4.0 ± 6.4
act. HCO_3^- (mmol/l)	23.5 ± 2.5	23.6 ± 4.9	21.5 ± 4.3	22.9 ± 5.4

$^*P < 0.05$

The three prematurely-born calves weighing between 18 and 26 kg were extracted between the 240th and 256th days of pregnancy with moderate assistance. One of them was suffering from mild, and two from severe, respiratory and metabolic acidosis. All three died within 24 h.

Discussion

Three to 4 hours after traction and 4 to 5 h after Caesarean section, newborn calves suffer from a distinct form of respiratory and metabolic acidosis. The acidosis of calves delivered by traction was shifted in a respiratory direction as a result of the higher pCO_2 values.

Maurer–Schweizer et al. (1977*b*) published findings agreeing with the above-mentioned in their report on the acid–base balance of newborn calves born *per vias naturales* and by Caesarean section.

The differences between the acid–base balance of all the calves (traction with moderate or intensive assistance, heifer-calves and cow-calves born in an anterior or a posterior presentation) indicate that the period of time during which the offspring passes through the birth canal, and within this period the compression of the umbilical cord, is of decisive importance. The above statement is supported by our results as there are no significant differences between the acid–base balance of the newborn calves of primi- or multiparous cows removed by Caesarean section. If the assistance was intensive, the pO_2 values were significantly higher immediately after parturition than if the assistance was moderate. This may be explained by the fact that in the case of hip-lock flexion, after cleaning the airways, we initiated breathing before traction was continued. Death within 24 h of premature calves may be caused by immaturity of the lungs (respiratory distress syndrome), but further examination is necessary (Kerpel–Fronius et al., 1978).

The acid–base balance of newborn calves is labile and can easily be shifted towards acidosis (Ammann et al., 1974; Eichler–Steinhauff, 1977; Massip, 1980; Mülling, 1974; Waizenhöfer, 1977). At birth, coinciding with the start of respiration, vasoconstriction is stopped and the accumulated acids enter the circulation. This may be the explanation for the further decrease of pH values and metabolic variables in the 10th min after calving (Bodenberger, 1979; Maurer–Schweizer et al., 1977*a*; Mülling et al., 1972; Waizenhöfer, 1977). According to our results, these decreases in pH and metabolic values are higher in case of intensive assistance with an anterior presentation and traction with a posterior presentation than in case of the other groups (Table IV).

In the case of a complicated calving, the mode of assistance should be chosen by taking into consideration the economic point of view, as well as

Table IV

Differences of average values of blood samples taken immediately post partum and 10 min later

Parameters	Anterior presentation				Posterior presentation	
	Traction		Caesarean section			
	Moderate ass.	Intensive ass.	Primiparous	Multiparous	Traction	Caesarean section
pH	−0.007	−0.086	−0.030	−0.003	−0.068	−0.015
pCO_2 (kPa)	−0.6	+0.7	0.0	−0.5	+0.5	−0.1
pO_2 (kPa)	+0.9	−0.5	+1.1	+0.7	0.0	+0.3
BE (mmol/l)	−1.8	−3.2	−1.7	−1.1	−2.8	−0.7
EBE (mmol/l)	−2.6	−2.4	−1.4	−1.3	−2.8	−0.7
act. HCO_3^- (mmol/l)	−2.2	−2.2	−1.5	−1.4	−2.0	−0.7

the aim that acid–base balance of newborn calves should be disturbed as little as possible and that asphyxia should be avoided.

If this is not possible, newborn calves with asphyxia should be treated adequately (Mülling, 1974; Eigenmann et al., 1982; Szenci, 1981; Walser and Maurer–Schweizer, 1978).

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EFFECT OF SEASON AND BACTERIAL CONTAMINATION ON SEMEN QUALITY, FREEZABILITY, AND FERTILITY OF HUNGARIAN SIMMENTAL ARTIFICIAL INSEMINATION BULLS

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Ejaculates collected from 100 Hungarian Simmental bulls, 3–5 years old, were used to investigate the effect of season on semen quality and freezability (400 ejaculates/season). Summer and winter samples were subjected to bacterial counting after freezing. Heat resistance and fertility per cent of the samples were also determined.

Season significantly affected the ejaculate volume, mass motility ($P < 0.05$), progressive motility and heat resistance ($P < 0.01$). Ejaculate volume and motility were superior in spring, and sperm concentration and number of sperm per ejaculate in autumn, as compared to the other seasons. Percentage of ejaculates with $<10^3$, $>10^3-5\times10^4$, $>5\times10^4-10^5$, $>10^5-1.5\times10^5$, $>1.5\times10^5-2\times10^5$ and $>2\times10^5$ viable bacteria/ml was 25, 20, 26, 13, 4 and 12 in winter, and 26, 16, 30, 10, 6 and 12 in summer, respectively. Sperm motility, heat resistance and fertility per cent were significantly influenced by bacterial contamination. No considerable variations were observed in the pH of semens containing different numbers of bacteria.

Keywords. Seasonal variation, bacterial contamination, semen quality, fertility, AI bulls.

The effect of season on bull semen has been studied by several investigators including Pavlicenko (1965), Milicevic (1965), Müller and Szilagyi (1965), Porwal et al. (1977) and Smirnov and Kruglyak (1980). Some of the results are contradictory, presumably due to differences in breed or environmental conditions. However, such studies seem to be lacking for Hungarian Simmental bulls.

Rózsa (1950) found very large numbers of viable bacteria in semen from fertile healthy bulls. Jacquet and Steig (1967) and Schafer et al. (1976) isolated various pathogenic and nonpathogenic organisms from microbial cultures of liquid nitrogen. Macpherson and Fish (1954) found *Mycobacterium tuberculosis* surviving in frozen semen. Krolinski (1978) demonstrated that the conception rate tended to decrease as the viable count in bull semen increased.

Materials and methods

One hundred Hungarian Simmental bulls, 3–5 years old, belonging to the Artificial Insemination Centre, Gödöllő, Hungary, were used. The bulls were kept under identical managemental and feeding conditions. Semen was

regularly collected at weekly intervals by means of an artificial vagina. One thousand and six hundred samples comprised the material for studying the effect of season on semen quality and freezability. Only the samples collected in the middle month of each season, i.e. January, April, July and October were used. The samples were examined for volume, mass and progressive motility (Müller and Szilagyi, 1965), sperm concentration, number of sperm per ejaculate, pH, and resistance to heat (Badawy, 1978; Ibrahim and Gaál, 1979; Ibrahim and Mészáros, 1980) after freezing in liquid nitrogen. The methods of semen examination were those routinely applied at the Artificial Insemination Centre, Gödöllő, Hungary, as described earlier (Ibrahim and Gaál, 1979). Representative samples from each bull were subjected to bacterial counting after freezing as described by Sutka (1980). Bacterial counting was performed in the summer and winter samples only. The samples were classified into 6 groups according to their bacterial content as follows: 10^3, $>10^3 - 5\times10^4$, $>5\times10^4 - 10^5$, $>10^5 - 1.5\times10^5$, $>1.5\times10^5 - 2\times10^5$ and $>2\times10^5$ viable bacteria/ml. The heat resistance and fertility per cent of the six semen groups were determined. The statistical analysis was conducted as described by Snedecor and Cochran (1968).

Results and discussion

Table I shows that the ejaculate volume was maximum (7.32 ± 0.21 ml) in the spring and minimum in the winter (6.20 ± 0.21 ml); the differences were statistically significant ($P < 0.05$). Pavlicenko (1965) and Smirnov and Kruglyak (1980) obtained for Simmental bulls minimum ejaculate volumes

Table I

Effect of season on semen quality and freezability of Hungarian Simmental A.I. bulls (n = 100)*

Semen traits	Winter	Spring	Summer	Autumn
Ejaculate volume (ml)	6.20 ± 0.21	7.32 ± 0.21	6.86 ± 0.26	6.84 ± 0.24
Number of sperm (×10⁹/ml)	1.49 ± 0.05	1.50 ± 0.04	1.49 ± 0.06	1.65 ± 0.05
Number of sperm (×10⁹ per ejaculate)	9.24 ± 0.01	10.98 ± 0.01	10.22 ± 0.02	10.43 ± 0.01
Mass motility	3.70 ± 0.09	3.96 ± 0.06	3.62 ± 0.08	3.58 ± 0.06
Progressive motility, %	66.80 ± 0.56	70.60 ± 0.52	66.40 ± 0.88	67.90 ± 0.79
Heat resistance at 0 h	39.00 ± 0.65	40.40 ± 0.67	35.30 ± 0.92	40.80 ± 0.66
after 1 h at 46 °C	36.10 ± 0.77	35.90 ± 0.75	21.10 ± 1.45	38.40 ± 0.64

* Each value represents mean ± SD for 100 bulls.

in winter, and maximum volumes in autumn. Müller and Szilagyi (1965) and Porwal et al. (1977) did not find significant seasonal differences in ejaculate volume in Simmental and Red Dane bulls, respectively.

Table I also shows that maximum sperm concentration was obtained in autumn, while in the other seasons almost identical sperm concentrations were found. The difference between autumn and other seasons in this respect was not significant. This finding agrees with data of Pavlicenko (1965), Müller and Szilagyi (1965), Badawy (1979), and Smirnov and Kruglyak (1980). On the other hand, Milicevic (1965) reported that low temperature affects sperm concentration. The results also indicated that the number of sperm per ejaculate was higher in spring, and it was the lowest in winter (Table I). Seasonal differences may be due to changes in testis activity. In summer an increased ejaculate volume was observed, but the number of sperm per ejaculate somewhat decreased. In other words, spermatogenesis was considerably more active in autumn, and the seasonal effect was more pronounced on the function of the accessory sex organs than on that of testes.

The mass and progressive motility were significantly ($P < 0.05$ and $P < 0.01$, respectively) influenced by season (Table I). The greatest motility was observed in spring and the least in summer. This means that high ambient temperature adversely affected sperm motility. Porwal et al. (1977) failed to observe this effect in Red Dane bulls.

Heat resistance was greatest in autumn, followed by spring, and the least resistance was found in summer (Table I). The differences in this respect were significant ($P < 0.01$). This finding is consistent with that obtained by Smirnov and Kruglyak (1980) in Russian Simmental bulls. It was concluded that in Hungary the semen samples most suitable for freezing are those produced in autumn and spring.

As shown in Table II, the distribution of bulls by the degree of contamination of their semen was approximately the same in winter and summer. The most common microorganisms identified in the semen samples were *Bacillus subtilis*, coliforms, *Staphylococcus aureus* and *Proteus* species. The percentage of progressively motile sperm significantly ($P<0.01$) varied between bull groups with different bacterial counts in their semen (Table II). Progressive motility tended to decrease as the bacterial count increased. This may be due to the possible competition for nutrients between bacteria and spermatozoa. Heat resistance showed a similar tendency, however, the effect of bacteria on heat resistance was considerably more pronounced than that on motility. As it was expected, freezability decreased as bacterial content increased, especially over 1.5×10^5 viable bacteria/ml semen. Semen pH slightly increased parallel with the elevation of the bacterial count, however, the differences were not significant. The change of semen pH is apparently caused by increased biological and metabolic activities of spermatozoa and microorganisms.

Table II

Effect of bacterial contamination on semen characters of Hungarian Simmental bulls (n = 100) in summer and winter

Bacterial count/ml	Number of bulls	Semen pH	Progressive motility (%)	Heat resistance	
				At 0 h	After 1 h at 46 °C
$<10^3$					
summer	26	6.40	69.20 ± 1.03	37.60 ± 2.30	35.40 ± 2.40
winter	25	6.40	69.20 ± 0.62	42.40 ± 0.90	39.40 ± 1.20
$>10^3-5\times10^4$					
summer	16	6.40	68.40 ± 1.18	37.80 ± 0.60	32.50 ± 2.60
winter	20	6.41	69.00 ± 0.86	40.80 ± 0.70	38.80 ± 1.40
$>5\times10^4-10^5$					
summer	30	6.41	62.80 ± 2.37	35.30 ± 2.20	26.50 ± 2.60
winter	26	6.40	66.00 ± 0.72	39.00 ± 0.70	29.60 ± 2.00
$>10^5-1.5\times10^5$					
summer	10	6.52	58.50 ± 5.63	34.00 ± 3.90	20.50 ± 5.10
winter	13	6.49	61.20 ± 2.78	30.40 ± 3.90	22.70 ± 4.10
$>1.5\times10^5-2\times10^5$					
summer	6	6.50	64.20 ± 1.54	36.70 ± 3.30	16.70 ± 4.70
winter	4	6.53	67.50 ± 1.44	28.80 ± 9.60	23.80 ± 8.00
$>2\times10^5$					
summer	12	6.50	66.50 ± 1.43	36.90 ± 1.30	18.50 ± 3.40
winter	12	6.48	62.10 ± 2.64	28.80 ± 5.10	10.40 ± 3.70

In his studies on Polish Black and White and Charolais bulls, Krolinski (1978) found that the conception rate was 72.7, 76.2, 72.3, 69.6 and 68.3% for semen samples containing $<5\times10^4$, $5\times10^4-2\times10^5$, $2\times10^5-4\times10^5$,

Table III

Effect of bacterial contamination of semen on fertility of Hungarian Simmental bulls

Bacterial count/ml	Number of inseminated cows	Number of conceived cows	Unconceived cows	Fertility %
$<10^3$	17,587	9,152	8,435	52.04
$>10^3-5\times10^4$	10,361	5,204	5,157	50.22
$>5\times10^4-10^5$	18,031	8,617	9,414	47.79
$>10^5-1.5\times10^5$	6,602	3,232	3,370	48.95
$>1.5\times10^5-2\times10^5$	5,342	2,662	2,680	49.64
$>2\times10^5$	40	13	27	32.50

4×10^5 — 10^6 and more than 10^6 bacteria/ml. Our present findings (Table III) agree with these results. There was no significant difference in the fertility per cent obtained for the different groups, with the exception of the group with a bacterial count of $>2\times10^5$/ml of semen, which showed significantly decreased fertility.

We can conclude from the present results that the semen quality and freezability of Hungarian Simmental bulls were significantly affected by the season. Best semen quality was found in spring and autumn and worst in winter. This is in agreement with the results of most investigators. The increase of the bacterial content to a level over 2×10^5 bacteria/ml of semen was accompanied by a remarkable decrease in fertility percentage. Semen quality was also considerably influenced by the increase of bacterial counts in the semen, especially if viable bacterial count exceeded 1.5×10^5/ml. Bacterial content can also affect freezability of the semen.

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STUDIES ON THE EFFECT OF PREMEDICATION ON ELECTROANAESTHESIA IN BUFFALO CALVES

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The efficacy of premedication on electroanaesthesia was studied on six buffalo calves of different ages and weights. These were compared with non-premedicated animals given electroanaesthesia. Premedication with triflupromazine-HCl reduced the quantity of mA required more than premedication with chlorpromazine-HCl.

Keywords. Premedication, triflupromazine-HCl, chlorpromazine-HCl, electroanaesthesia, buffalo calves.

Electroanaesthesia is one of the safest means of producing general anaesthesia in ruminants as chemical anaesthesia is beset with problems such as ruminal tympany, regurgitation and aspiration.

Alternating electric current has been used as an anaesthetic agent for various surgical procedures in cattle (Short, 1964, 1967, 1974) and in buffaloes (Rao and Rao, 1978). The use of premedicants resulted in minimal undesirable reactions during induction of electrical anaesthesia (Short, 1967). Reduction in the quantum of current requirement following tranquillization has been reported in dogs (Smith et al., 1967) and buffaloes (Rao and Rao, 1979).

In the present investigation, efficacies of two preanaesthetics, namely triflupromazine hydrochloride (Siquil) (Sarabhai Chemicals, Bombay) and chlorpromazine hydrochloride (Largactil) (May and Baker, Bombay) have been compared on the course of electroanaesthesia in buffalo calves.

Materials and methods

Six apparently healthy male buffalo calves of 1 ½ to 3 years of age and 40 to 114 kg body weight were used. The animals were cast and secured in right lateral recumbency prior to the experiment. Each animal was subjected to repeated trials at 72-h intervals.

In group I electroanaesthesia was produced without premedicant. In group II triflupromazine-HCl, and in group III chlorpromazine-HCl was used intravenously as premedicant at a dose rate of 0.25 mg/kg body weight about 10 min prior to electroanaesthesia. The animals were anaesthetized for one

hour by step-by-step method with bitemporal electrodes by the electronic anaesthetizer described by Rao and Rao (1978). Abdominal muscle relaxation was taken as the end-point of surgical anaesthesia, at which time Eger's test was performed (Smith et al., 1967). The milliamperage (mA) and voltage (V) and the resulting symptoms were recorded. The data were subjected to Student's *t* test as described by Snedecor and Cochran (1967) to find the level of significance, using 2-tailed test.

Results and discussion

The various observations made on electroanaesthesia without and with premedication are given in Table I. Slight discomfort was observed during induction of anaesthesia in animals without premedication. This was not

Table I

Effect of premedication on electroanaesthesia in buffalo calves

S. No.	Age (years)	Weight (kg)	Non-premedicated group			Triflupromazine-HCl group			Chloropromazine-HCl group		
			Volts	mA	Pain on Eger's test	Volts	mA	Pain on Eger's test	Volts	mA	Pain on Eger's test
1	1½	53	8	65	+	8	55	—	8	60	+
2	2½	114	10	80	+	10	55	—	10	60	—
3	2	48	12	65	+	12	50	—	12	60	+
4	1½	40	12	65	—	12	40	—	12	45	—
5	3	92	12	70	—	12	50	—	12	60	—
6	3	108	10	80	—	10	55	—	10	60	—
Mean ± SE		75.83 ± 13.209	10.66 ± 1.739	70.83 ± 3.005		10.66 ± 1.739	50.83*** ± 2.387		10.66 ± 1.739	57.50** ± 2.499	

**P < 0.01
***P < 0.001
+Present
—Absent

observed in animals premedicated with either triflupromazine-HCl or chlorpromazine-HCl. Thus, premedication produced smooth induction, which was in agreement with the observations made by Short (1974) and Rao and Rao (1979).

The mean quantity of mA required by non-premedicated animals was 70.83 ± 3.005 (range: 65 to 80), while animals premedicated with triflupromazine-HCl

azine-HCl required on the average 50.83 ± 2.387 mA (range: 40 to 55). Animals premedicated with chlorpromazine-HCl required on the average 57.50 ± 2.499 mA (range: 45 to 60). This shows that premedication reduced the mA required to produce anaesthesia. The reduction was highly significant ($P < 0.001$) in the triflupromazine-HCl group, and significant in the chlorpromazine-HCl group ($P < 0.01$) as compared to non-premedicated animals. However, no significant variation was observed in mA requirement between the two premedicated groups.

All the animals in the three groups showed muscular relaxation along with lachrymation and salivation or a wet muzzle. Tympany of the rumen was not observed. Slight movement of the limb during Eger's test was observed in 3 animals of the non-premedicated group, and in 2 animals of the chlorpromazine-HCl group.

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STUDIES ON THE BIOLOGY AND PATHOLOGY OF THE COMMON CARP PARASITE *MYXOBOLUS BASILAMELLARIS* LOM ET MOLNÁR, 1983 (MYXOZOA: MYXOSPOREA)

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Myxobolus basilamellaris Lom et Molnár, 1983 is a frequent parasite of common carp fry and one-summer common carp cultured in fish farms. In the development of the parasite in the fish, a two-peaked seasonality can be observed. The parasite occurs in 2 to 4 months old fry in summer, while in one-summer carp the cysts appear after overwintering, in the early spring months, and by the beginning of summer they become evacuated. *M. basilamellaris* is a host- and organospecific parasite. Its cysts develop at characteristic locations, on the base of the gill filaments or in the inside of the cartilaginous gill arch, forming outer or inner cysts which often communicate. The development of cysts always starts from the connective tissue which covers the basis of the cartilaginous gill rays supporting the filaments. *M. basilamellaris* is a moderately pathogenic parasite species. Mortality due to this species has not been observed so far, although the circulatory disorder caused by the parasite through deforming the blood vessels of gills is significant.

Keywords. Myxosporea, biology, pathology, common carp parasite.

During the regular animal health survey conducted in Hungarian fish farms, a *Myxobolus* species was found in common carp fry and one-summer common carp, on the base of the gill filaments, attached directly to the cartilaginous structure of the gill arch or developing in its cavity, in cysts of the size of a pinhead. This *Myxobolus* species was not identifiable with any of the *Myxobolus* and *Myxosoma* species described previously in carp. On the basis of its morphological properties and characteristic location, Lom and Molnár (1983) described this parasite as a new species, *Myxobolus basilamellaris*.

The number of *Myxosoma* and *Myxobolus* species parasitizing carp is extremely large. Of these, Shulman (1962) described 16 species in the Soviet Union, and Akhmerov (1960) found 8 species, among them 5 new ones, in common carp living in the River Amur. During his studies on fish living in the River Surkhandaria in Central Asia, Allamuratov (1974) reported the occurrence of 10 *Myxobolus* and *Myxosoma* species in common carp. According to Walliker (1968), the names *Myxobolus* and *Myxosoma* can be considered synonymous. Recently Yakovtshuk (1979) has increased the number of *Myxobolus* spp. parasitizing common carp with *M. musseliusae* found in the gill filaments. In Shulman's monograph (1966), the majority of the species

described in common carp are not regarded as host- and organospecific parasites. In this monograph, different fish species are indicated as hosts, and different organs as possible locations for the same *Myxobolus* species. In Mitchell's opinion (1977), both strictly host- and organospecific species and less specific ones can be found among Myxosporea.

The present paper reports on the dynamics of occurrence, seasonal phases of cyst development and pathological significance of *M. basilamellaris*, based on the results obtained during the systematic dissection of fish and by histological examinations.

Materials and methods

Since the first observation of *M. basilamellaris* in 1978, we have systematically performed dissection of carp fry and one-summer carp of a fish farm in Eastern Hungary. The survey was conducted from March up to the end of November. Occasionally, older carp and herbivorous fishes kept in the same pond (grasscarp, silver carp, bighead) were also examined. Three to 5 fish were sampled on each occasion. For routine histological examination, samples were taken from the gills, inner organs and muscles of the fish, were placed into 10% buffered formalin, and frozen sections were prepared from them. Organ samples taken from infected or suspect fish, as well as from some control ones, were fixed in Bouin's solution, embedded in paraffin and sectioned. To determine the location of cysts more precisely, transversal sections were made from some gills, in addition to the usual longitudinal ones. The majority of the sections were stained with haematoxylin and eosin, although Giemsa's, Kossa's, Farkas–Mallory's and van Gieson's staining techniques were also used.

For routine diagnostic purposes, fish sent to the laboratory from other fish farms of Hungary were also examined systematically and used for comparative purposes.

Results

Macroscopic examinations. Infection was always observed first in about two months old carp fry, in the second half of July. At that time, white papules of the size of a pinhead were seen, sometimes also with the unaided eye, on the base of the gill. These proved to be *Myxobolus* cysts containing spores (Fig. 1). At the same time, the presence of young developmental stages was demonstrated histologically in some symptom-free fish. At the end of August, only spore-containing cysts were found, and from September onwards infected individuals could no longer be detected. Infection was demonstrated again in the stock early in March, after the ice had passed, when spore-contain-

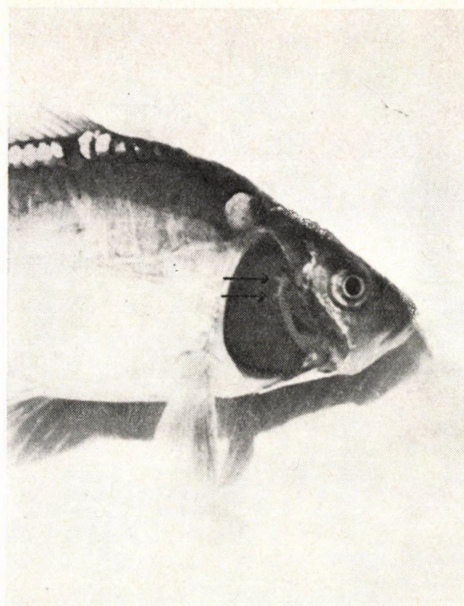

Fig. 1. Anterior part of a one-summer carp. The operculum is removed. Note the two white *Myxobolus basilamellaris* cysts (arrow) on the gill arch, on the side facing the gill filaments. Natural size

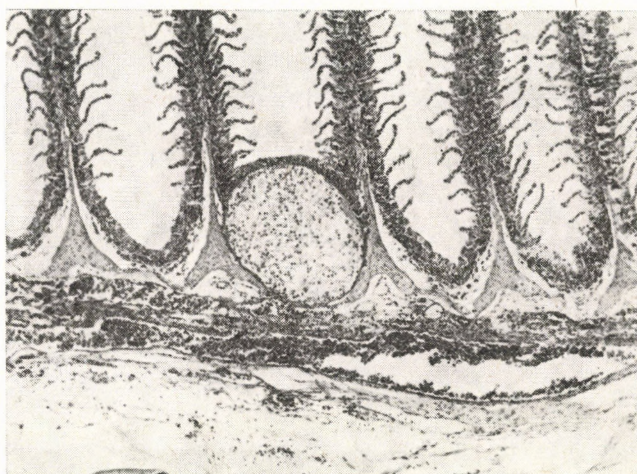

Fig. 2. Longitudinal section of an outer *M. basilamellaris* cyst located at the base of the gill filaments. H–E. ×75

ing cysts were observed again. This infection gradually subsided up to the end of April and disappeared in May. Afterwards, infection did not occur on older fish at all. Infection was diagnosed in all ponds of the fish farm studied, although its rate varied from pond to pond (3–45%). In intensive infection, the number of cysts per gill arch reached 10 to 18.

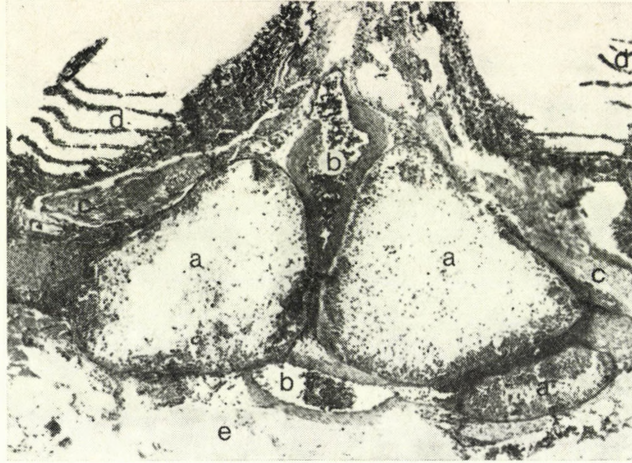

Fig. 3. (a) *M. basilamellaris* cysts in the cavity of the gill arch; (b) blood vessels of the gill; (c) cartilaginous base of the gill filaments; (d) remnants of secondary (respiratory) lamellae; (e) cavity of the gill arch. Transversal section, H–E. ×75

Fig. 4. (a) *M. basilamellaris* cyst having both an outer and an inner sack; (b) respiratory lamellae; (c) cavity of the gill arch; (d) cartilaginous base of the gill filaments. Longitudinal section, H–E. ×75

The presence of *Myxobolus basilamellaris* was established also in some other fish farms of Hungary. However, the latter farms were not subjected to thorough survey. Cysts of *M. basilamellaris* were not found in herbivorous fishes, not even if these were kept in polycultures.

Histological examinations. M. basilamellaris cysts were found in two adjoining, strictly specific, locations. The cysts referred to as "outer" cysts were located at the base of the gill filaments (Fig. 2), while the "inner" ones in the cavity of the cartilaginous gill arch (Fig. 3). Both the outer and the inner cysts were closely connected with the base of the cartilaginous ray of the gill filaments, which structure constituted also the vaulting of the cavity of the gill arch. The majority of cysts consisted of two sacks, one located at the basis of the gill filaments and the other within the gill arch. The two sacks

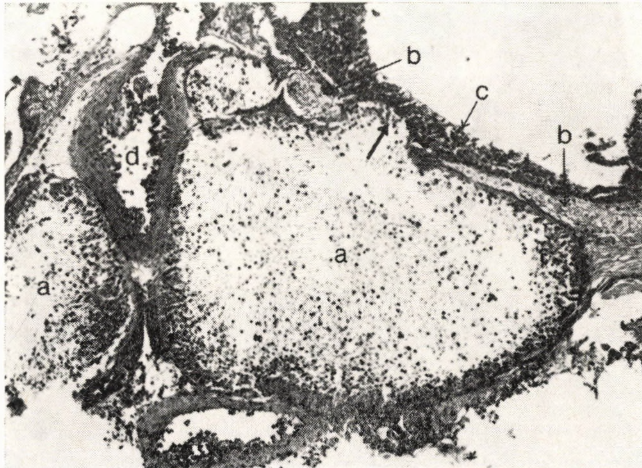

Fig. 5. Presumed mode of evacuation of an inner cyst. (a) cysts; (b) cartilage broken through or pushed aside; (c) epithelium; (d) blood vessels. Cross-section, H–E. ×120

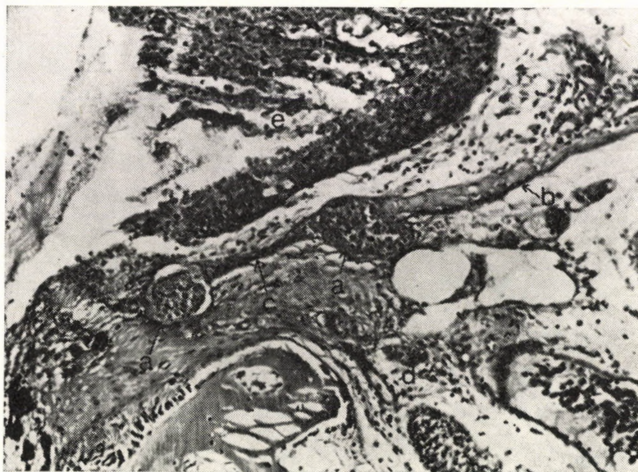

Fig. 6. Initial phase of cyst development. Young cysts (a) in the perichondral connective tissue (c) of the cartilaginous base (b) of gill filaments; (d) cavity of the gill arch; (e) respiratory lamellae. Cross-section, H–E. ×160

were connected by a narrow isthmus which ran between the cartilaginous bases of gill filaments and passed through the connective tissue layer surrounding the blood vessels and nerves (Fig. 4). However, even after having examined large numbers of samples, we are unable to establish obviously whether there are cysts consisting only of outer and inner sacks and lacking the isthmus, or the absence of communication results only from the imperfection of histological technique. In a transversal section of the gill arch it can be seen (Fig. 5) that the widening out of a mature inner cyst pushes aside the cartilaginous base, and the cyst protrudes outwards. This figure indicates that inner cysts probably lack the isthmus, and shows the probable mechanism of spore evacuation. The youngest cysts were found in the perichondral connective tissue covering the cartilaginous bases of gill filaments (Fig. 6). Both the outer and the inner cysts, even in fully matured stage, were demarcated from the cartilaginous cells only by a single cell layer of connective tissue (Fig. 7). The same single-layered connective tissue demarcated the cysts from the surrounding cellular elements also in parts where they were not contiguous with cartilage cells (Fig. 7). The connective tissue character of the cells constituting the capsule of the cyst was confirmed by the specific staining techniques (van Gieson's and Farkas–Mallory's staining). Both the young and the old cysts contained a narrow eosinophil ectoderma 5 μm in thickness. Within the ectoderma, in the young cysts a thin layer of developmental stages, primarily pansporoblasts, with deeply-staining nuclei was demonstrable (Fig. 7). The central part of the cysts was filled with spores even in young cysts. In more developed cysts, the ectoderma directly adjoined the spores.

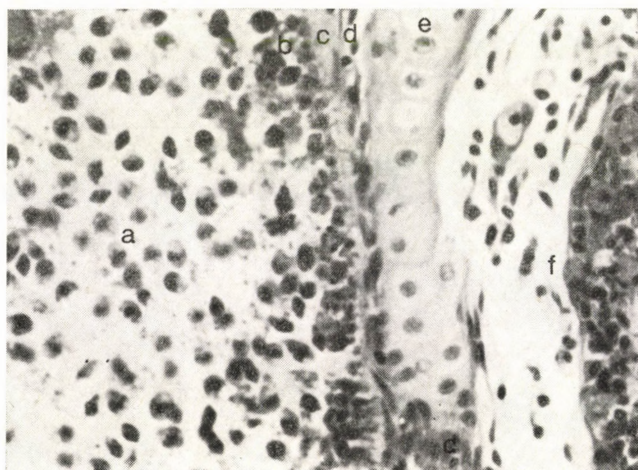

Fig. 7. Relation of the cyst to the cartilaginous structure of the gill filament. (a) spores; (b) developmental stages; (c) ectoplasm of the cyst; (d) perichondral connective tissue cells; (e) cartilaginous structure; (f) cavity of the gill arch. H–E. ×350

Fig. 8. First type of reparation of disintegrated and partly evacuated inner cysts. Granulation tissue (b) penetrates into the cavity of the disintegrated cyst (a), in between the retained spores. Mature spores in a not yet disintegrated cyst (c); (d) cartilaginous base of the gill filaments. Longitudinal section, H–E. ×75

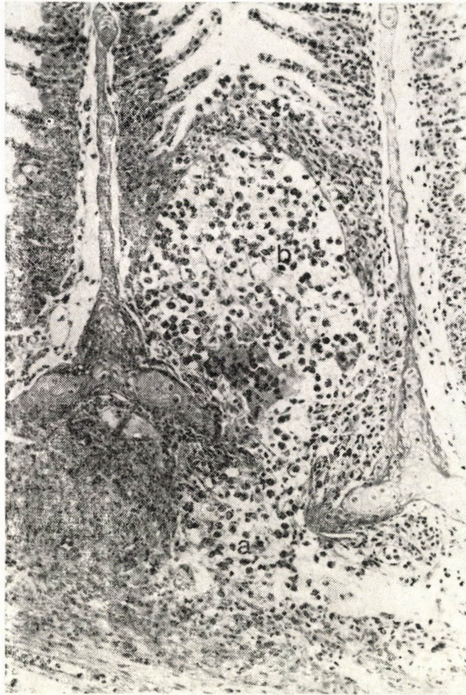

Fig. 9. Reparation of partly evacuated, communicating outer and inner cysts. Loose connective tissue proliferated around the spores retained in the place of the inner (a) and outer (b) cyst, and the spores gathered into lacunas. Longitudinal section, Farkas–Mallory's stain ×75

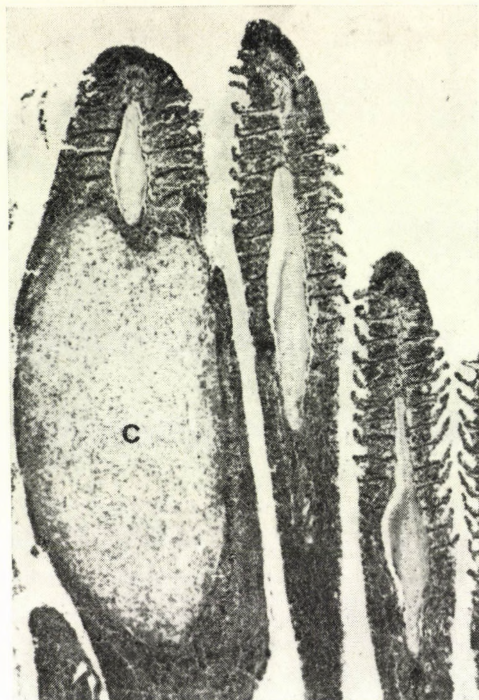

Fig. 10. Cyst of *Myxobolus dispar* (C) on the apical part of the gill filament. Longitudinal section, H–E. ×75

After the burst of the cyst, spores retained in the tissues were generally found only within the gill arch. Reparation of tissues took place without appearance of inflammatory cells. Two types of regeneration were discernible. In the first type, the granulation tissue consisting of large cells abundant in plasma penetrated into the decomposed cyst as a uniform mass (Fig. 8), while in the second type of regeneration the spores retained in the cyst were surrounded by loose connective tissue and gathered into lacunas containing a few spores (Fig. 9). Spores isolated in this manner showed signs of degeneration.

Associated infections. Gill necrosis, the damage regarded primarily as a condition caused by adverse environmental factors, was frequent in the stocks studied. *Dactylogyrus*, primarily *D. extensus*, infection of the fish was demonstrated in almost all cases. Occasionally gill sphaerosporosis, sanguinicolosis and *Myxobolus dispar* infection were also diagnosed. *Myxobolus basilamellaris* and *M. dispar* infections were easily distinguished on the basis of parasite location and spore morphology, since the latter gave rise to the development of cysts at the ends of gill filaments (Fig. 10) and resulted in breaking off of filaments.

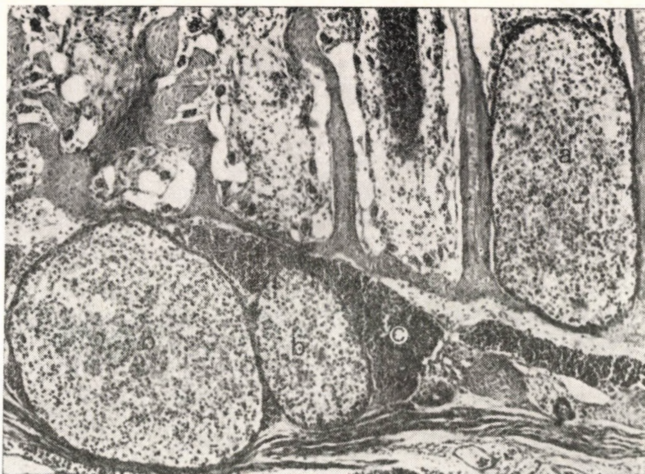

Fig. 11. Outer (a) and inner (b) cysts in the gill of a carp. Note the congestion (c) in the gill arch cavity blocked by cysts. Longitudinal section, H–E. ×75

Aetiology and pathogenesis. Mortality due to myxobolosis was not observed in the infected stocks, not even in severe infection. The occasional absence of some gill filaments was always attributable to associated infections, primarily to gill necrosis and dactylogyrosis. Breaking off of gill filaments was not observed even in more severe infection. However, the local pathogenic effect of *M. basilamellaris* cysts was easily demonstrable. The growing outer cysts lifted the basal part of gill filaments from their base and deformed the neighbouring respiratory lamellae, thereby leading to reduction of the respiratory surface (Figs 2 and 4). The inner cysts filled the major part of the gill arch cavity (Fig. 3), deformed and narrowed down the blood vessels and nerves passing through the cavity, causing congestion (Fig. 11). At the sites of cyst evacuation and near the isthmus demarcating the outer and inner cysts, deformation of the cartilage layer was also seen (Figs 3 and 5). General clinical symptoms were not observed in the fish.

Discussion

On a morphological basis, Lom and Molnár (1983) have proved that *M. basilamellaris* is a new, previously unknown, carp parasite. Our present investigations have shown the major biological characteristics of the parasite. A survey of several hundred fish has proved that this *Myxobolus* species is strictly organospecific and it occurs exclusively on the gills, having special affinity to the fibrous perichondral cells constituting the base of the cartilaginous arch of gill filaments.

So far, *Myxosoma cartilaginis* parasitizing Centrarchidae fishes has been the only species known to develop in the gill arch (Hoffman et al., 1965). However, this species, similarly to *Myxosoma cerebralis* studied by Halliday (1973), starts to develop among the cartilage cells, and not on their surface. Cyst development starting from the cartilage membranes has been described in detail by Molnár (1982) who found *Thelohanellus nikolskii* cysts surrounded by perichondral cells of the fin rays. The composition of the two cyst walls of host origin differs significantly, since the cartilaginous capsule seen around *Thelohanellus* cysts is absent from those of *M. basilamellaris*. This difference obviously results from the different nature of the two cartilages; the cartilage of gill filaments is a hyaline cartilage consisting of cartilage cells, while that of the fin rays is produced by the perichondral cells surrounding them. This queries the nature of the cells surrounding *M. basilamellaris* cysts, i.e. whether these cells can be regarded as perichondral cells.

Organospecific *Myxobolus* species have been rarely mentioned in the literature. *M. dogieli* living in the myocardium of common carp (Shulman, 1966; Bauer et al., 1977) and *M. cerebralis*, which lives in the cartilages of the skull of salmonids, belong to these. The observations of certain authors (Ivasik, 1953; Lucký, 1965; Bauer et al., 1981) and our results also suggest that the exclusive location of *M. dispar* is the apical and central part of the gill filaments, in spite of the fact that it had earlier been reported to occur in several organs.

Our knowledge concerning the host specificity of Myxosporea is insufficient because infection experiments are unaccomplishable. The most reliable observations were made during transcontinental fish transportations. It was proved during these transportations that *M. cerebralis* introduced to America was able to infect all members of Salmonidae (Hoffman and Putz, 1969; O'Grodnick, 1979), and thus it was regarded as a parasite with a relatively wide host range. On the other hand, *Myxobolus pavlovskii* and *M. drjagini*, the parasites of the *Hypophthalmichthys* species introduced to Europe, can find the conditions essential for their life only in their original hosts, *H. molitrix* and *H. nobilis* (Molnár, 1979; Yakovtshuk, 1981). According to our present knowledge, *M. basilamellaris* belongs to the latter type of *Myxobolus* species.

As regards the development of Myxozoa, two types have been distinguished in the literature. In the first type, spore production takes place continuously in different seasons, in a relatively short time. The development of *M. pavlovskii* belongs to this type (Lucký, 1978; Molnár, 1979). The reproduction of other species, e.g. *Hoferellus cyprini*, *Myxosoma cerebralis*, *Myxobolus drjagini* is characterized by a seasonal cycle covering a year (Plehn, 1924; Shulman, 1966; Wu Baohua et al., 1979). The development of *M. basilamellaris* shows a two-peaked seasonality. The developmental cycle of the parasite runs its course within a short time in carp fry infected soon after hatching, and

M. basilamellaris produces mature spores in two months' time. In addition to carp fry, an infestation can be demonstrated also in one-summer carp in the spring. In the latter case, the early development of the cysts takes place in the winter months, and they develop slowly, obviously due to the low water temperature. Infection of this type has also been described in the literature: Shulman (1966) reported similar cases for *Chloromyxum fluviatile*, *Myxobilatus legeri* and *Myxidium lieberkühni*.

The cellular response of the host organism is negligible, since the cyst is surrounded by only one cell row of connective tissue. The studies of Dyková and Lom (1978) showed that a marked tissue response accompanied by granuloma formation started only after complete maturation of spores. During this response, granulation tissue filled the place of cysts and eliminated the retained spores. Outer cysts of *M. basilamellaris* located at the base of gill filaments presumably evacuate their contents to the outside world easily since they are surrounded by a single cell row of connective tissue origin and by a narrow epithelial cell layer only. Spores may get to the outworld from the inner sack having an isthmus in a similar manner, thus, reparation may take place without marked granulation tissue formation. The mode of evacuation of cysts having only an inner sack is not known yet. Certain observations indicate that such cysts can also evacuate their contents to the outside world, past the base cartilages of gill filaments (Fig. 5). The majority of spores, however, are retained in the cavity of the gill arch and become surrounded by granulation tissue. In our cases the granulation tissue did not contain inflammatory cells or round cells.

The results obtained on the pathogenicity of the parasite are insufficient. Cysts developing at the base of the gill filaments neither cause severe damages in the respiratory lamellae nor lead to the breaking off and lasting deformation of gill filaments. The role of the cysts or parts of cysts developing in the gill arch cavity is more significant, since these impair the functioning of blood vessels and nerves of this area. Due to the insufficient blood circulation, severe *M. basilamellaris* infection may be a significant factor in impairing the general resistance of the organism. It cannot be excluded that secondary pathogenic agents *(Dactylogyrus, Flexibacterium)* may establish themselves more easily on the gill filaments insufficiently supplied with blood, thereby resulting in secondary invasion or infection.

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MORPHOLOGICAL AND HISTOCHEMICAL STUDIES FOR THE EVALUATION OF SEVERAL IRON PREPARATIONS IN PIGLETS

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Gross pathological, histopathological, histochemical and electron microscopic procedures and blood analysis were used to compare the absorption and distribution of a peroral (Hemogen pulvis) and three parenteral (Chinofer 75 mg Fe/ml, Leodex and Master-mix) iron preparations in 5 and 21 days old suckling pigs.

Of the three dextran-type injection preparations, Leodex and Master-mix absorbed rapidly and efficiently, and was distributed uniformly in the organism, considerably elevating the iron concentration and total iron-binding capacity of the blood. Chinofer formed a "local iron depot" primarily at the site of injection and in the regional lymph nodes. Hemogen pulvis (ferrous oxalate) administered through a stomach tube resulted in sufficient iron concentrations in the blood. The age of the piglets did not influence the absorption and distribution properties of the four iron preparations studied.

Keywords. Iron preparations, morphological study, piglets.

Although iron supplementation given to sows cannot eliminate the iron deficiency syndrome of piglets, it may constitute an important part of the complex prevention. Therefore, iron supplementation administered to newborn piglets perorally or in injection form is indispensable. The aim of the present experiment was to study the absorption and tissue distribution of various iron preparations administered by different routes.

Materials and methods

Piglets. TETRA-S hybrid suckling piglets kept in a large-scale pig unit were used. Ten suckling pigs of the same litter were chosen for the experiment. The piglets were in an apparently identical developmental state. They were marked individually in a permanent manner.

Iron preparations. Chinofer inj. ad us. vet., 75 mg iron/ml; Master-mix inj. ad us. vet.; Leodex inj. ad us. vet.; ferrous (II) oxalate. Chinofer, Master-mix and Leodex were injected into the thigh muscle, whereas ferrous oxalate was administered through a stomach tube.

Two piglets were treated with each preparation, the one when 3 days old and the other when 19 days old. Each animal received 100 mg iron/kg body weight in a single dose. The treated piglets were exsanguinated in the

48th h after iron administration (i.e. when 5 and 21 days old) together with one untreated (negative) control in each case.

Histological and histochemical examinations. Samples were taken from the thigh muscle at the site of injection, from the subiliacal, mesenteric and cervical lymph nodes, liver and spleen. After fixation in formalin and embedding in paraffin, the sections were stained with haemalaun and eosin. In frozen sections prepared from the liver, Fettrot staining was used for the detection of lipids, iron was demonstrated by Turnbull's reaction, while dextran and glycogen by the PAS reaction. Gömöri's silver impregnation method was used for the examination of the reticular fibre network.

Electron-microscopical examination. One sample was taken from the liver of each animal. After double fixation in 2.5% glutaraldehyde and in 1% osmium tetroxide, both buffered with 0.1 M sodium cacodylate, and dehydration with ethanol, the sample was embedded in Durcupan ACM in the usual way. Ultrathin sections were counterstained with uranyl acetate and lead citrate, and examined in a Philips 201 CS electron microscope.

Chemical assays. The iron concentration and total iron-binding capacity of the blood plasma were determined. For this, blood samples were withdrawn from all animals before iron treatment and prior to slaughtering performed 48 h later, and were analysed using La Roche's diagnostic iron test. Photometry was performed in a Spektromom 204 apparatus, at a wavelength of 546 nm.

Results

At *necropsy* of the 5 and 21 days old piglets treated with Chinofer, the subcutaneous connective tissue and muscles of the thigh ipsilateral with the injection site showed intensive brownish-black discolouration and were slightly oedematous from the subiliacal to the perianal region. The ipsilateral subiliacal and iliacal lymph nodes were also brownish-black, swollen and succulent. On the other hand, neither the contralateral subiliacal and iliacal lymph nodes, nor lymph nodes of more remote regions showed notable discolouration. In animals treated with Master-mix or Leodex preparations, only mild yellowish-brown discolouration of the subcutaneous connective and muscle tissue of the thigh was observed. At the same time, all regional and visceral lymph nodes were consistently yellowish-brown and slightly swollen. The above changes were absent in both untreated control piglets and those treated with ferrous oxalate.

The *histological and histochemical examination* of the 5 and 21 days old piglets treated with Chinofer revealed the presence of large quantities of Turnbull- and PAS-positive granular and cloddish material around the injection site, primarily extracellularly in the connective tissue between muscle fibres

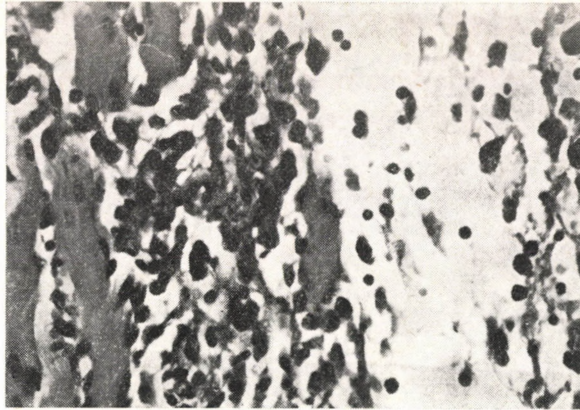

Fig. 1. Detail of the thigh muscle from a piglet treated with Chinofer 75 in a dose of 100 mg iron/kg body weight. Muscle fibres show Zenker's necrosis, serous and cellular infiltration by inflammatory cells, and appearance of fibroblasts. Haemalaun-eosin stain, ×400

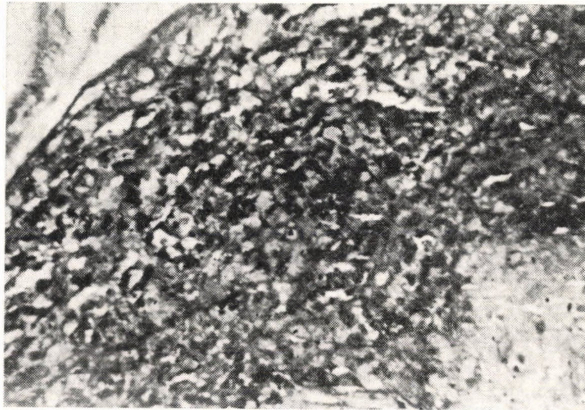

Fig. 2. Detail of subiliacal lymph node from a piglet treated with Chinofer 75 in a dose of 100 mg iron/kg body weight. The marginal sinus contains large amounts of iron-dextran. PAS reaction, ×240

and bundles and, in phagocytosed form, in the cytoplasm of histiocytes (macrophages). Occasionally muscle fibres showed Zenker's necrosis, serous and mixed-type cellular infiltration with different inflammatory cells (neutrophil granulocytes, lymphocytes, histiocytes and plasma cells), together with incipient proliferation of fibroblasts (Fig. 1). The major lymphatic vessels and the sinus system of the regional lymph nodes were filled with iron-dextran. Iron-dextran was present in the cytoplasm of the swollen endothelial and reticulum cells (macrophages), but most of it was seen extracellularly (Fig. 2).

In lymph nodes situated far from the injection site (mesenteric and cervical lymph nodes), iron-dextran was demonstrated only in small amounts

and intracellularly in the endothelial cells of sinuses and in the reticulum cells (macrophages). Iron-dextran was present also in reticulum cells of the red pulp of the spleen and in Kupffer cells of the liver. Liver cells of the 5 days old piglets contained large, while those of the 21 days old ones smaller amounts of glycogen. Lipids were not demonstrable in hepatic cells. The reticular network was unchanged in both 5 and 21 days old animals.

Only small quantities of Master-mix and Leodex were present in the thigh muscle of the 5 and 21 days old animals in the connective tissue between muscle bundles and fibres, dispersed or in the form of single clods or groups of granules extracellularly and in phagocytosed form in the cytoplasm of macrophages (Fig. 3). The muscle fibres were intact. A pronounced iron-

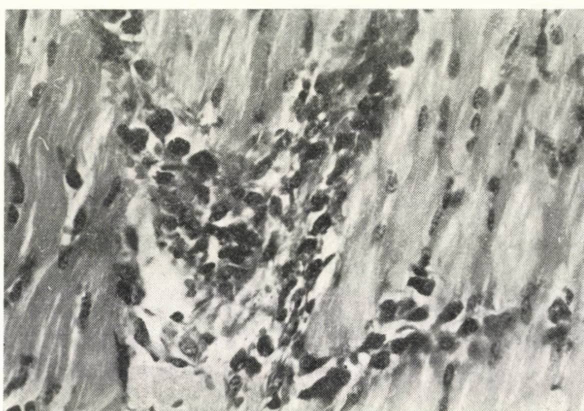

Fig. 3. Detail of the thigh muscle from a 5-day-old piglet treated with Leodex in a dose of 100 mg iron/kg body weight and killed by bleeding 48 h thereafter. Small amount of granular iron-dextran is present in the connective tissue. The muscle fibres are intact. Haemalaun-eosin, ×400

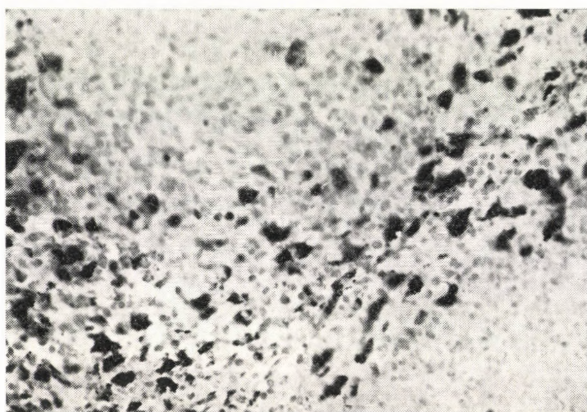

Fig. 4. Detail of spleen from a 5-day old piglet treated with Leodex in a dose of 100 mg iron/kg body weight and killed by bleeding 48 h thereafter. Iron-dextran is present in reticulum cells of the red pulp, around the Malpighian bodies. Turnbull's reaction, ×400

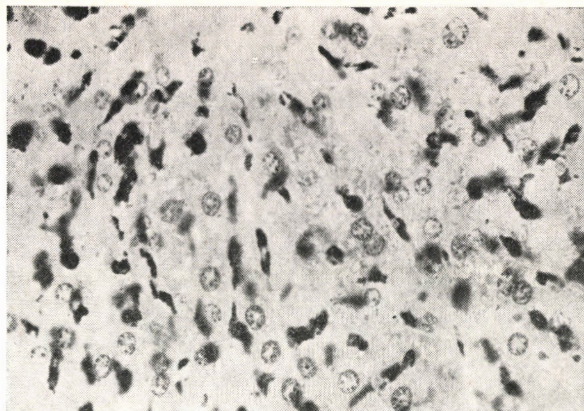

Fig. 5. Detail of liver from a 5-day-old piglet treated with Master-mix in a dose of 100 mg iron/kg body weight and killed by bleeding 48 h thereafter. Kupffer cells of the sinusoids contain iron-dextran. Turnbull's reaction, ×400

dextran deposition was observed primarily in reticulum cells and endothelial cells of sinuses in the subiliacal, mesenteric and cervical lymph nodes. In the subiliacal lymph node, iron-dextran was deposited also extracellularly. Reticulum cells of the red pulp of the spleen (Fig. 4) and Kupffer cells of the liver (Fig. 5) also contained iron-dextran.

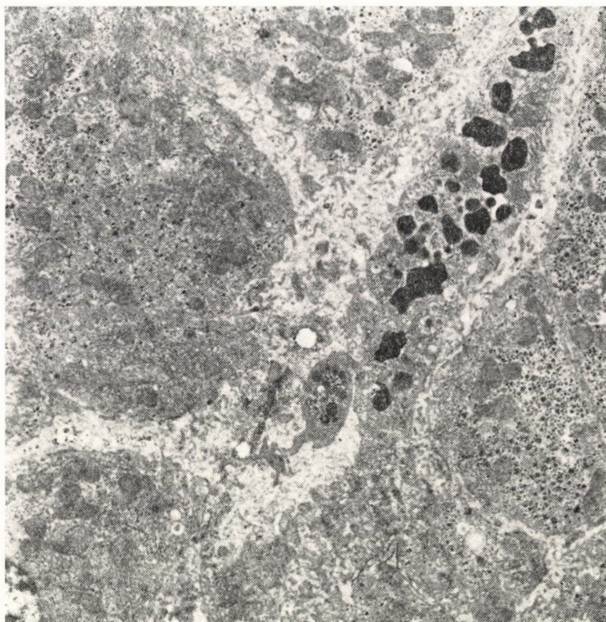

Fig. 6. Kupffer cell filled with phagocytosed iron, in the liver from a 5-day-old piglet treated with Chinofer. Electron micrograph (EM), ×6500

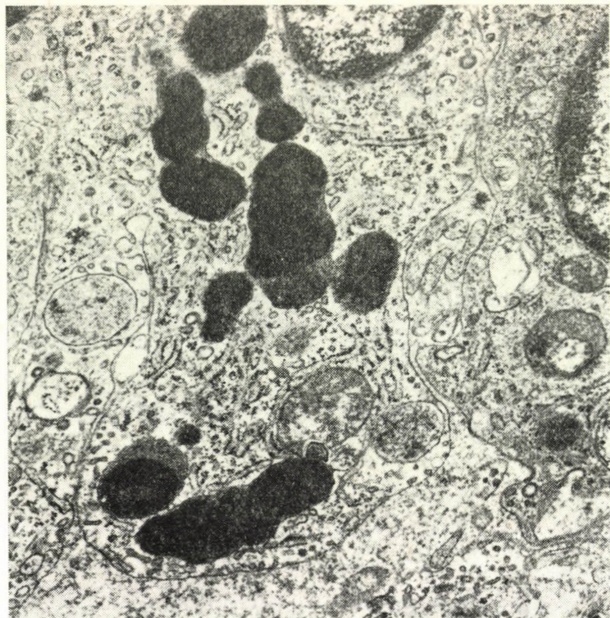

Fig. 7. Kupffer cell containing vast amount of phagocytosed iron, in the liver from a 5-day-old piglet treated with Leodex. EM ×22,000

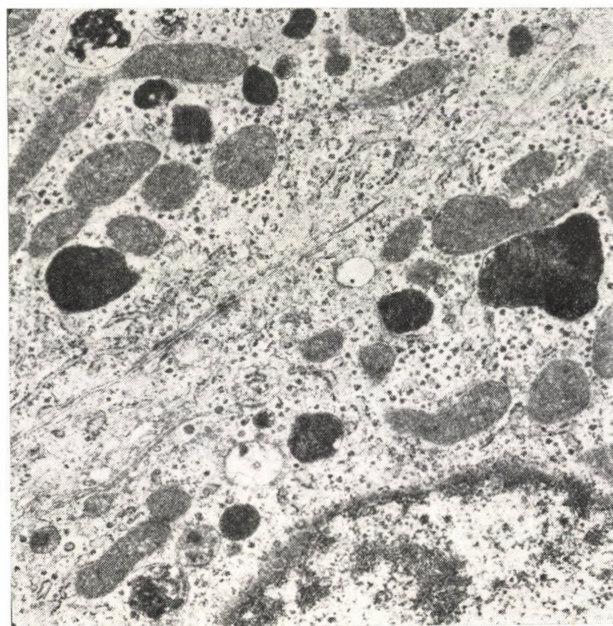

Fig. 8. Siderosomes in liver parenchymal cells from a 5-day-old piglet treated with Leodex. EM ×22,000

Liver cells of the 5 days old piglets contained more glycogen than those of the 21 days old ones also in this case, but lipids were absent in them. The reticular fibre network of the liver did not show pathological changes.

In the examined organs of untreated control piglets and of those treated with ferrous oxalate, iron was not demonstrable by the histochemical reactions neither in 5 nor in 21 days old animals.

Electron microscopically, the absorption of iron and its distribution in the examined organs of pigs were found to be of similar character after Master-mix and Leodex treatment. However, after Chinofer treatment both the absorption and the distribution of iron were different; iron showed uneven distribution in the liver of both 5 and 21 days old piglets. The endothelial and Kupffer cells contained vast amounts of phagocytosed iron, while considerably smaller amounts of iron were present in hepatocytes (Fig. 6).

After Master-mix and Leodex treatment, liver cells, endothelial and Kupffer cells contained numerous and large, electron dense vacuoles (iron-containing siderosomes) in both age groups (Figs 7 and 8).

A common characteristic of the three parenteral preparations was that after treatment iron was present in large vacuoles surrounded by a membrane in endothelial and Kupffer cells, as a finely granular dense substance. Parenchyma cells also contained such vacuoles, but apart from these, the absorbed iron was primarily located in secondary lysosomes, sometimes together with

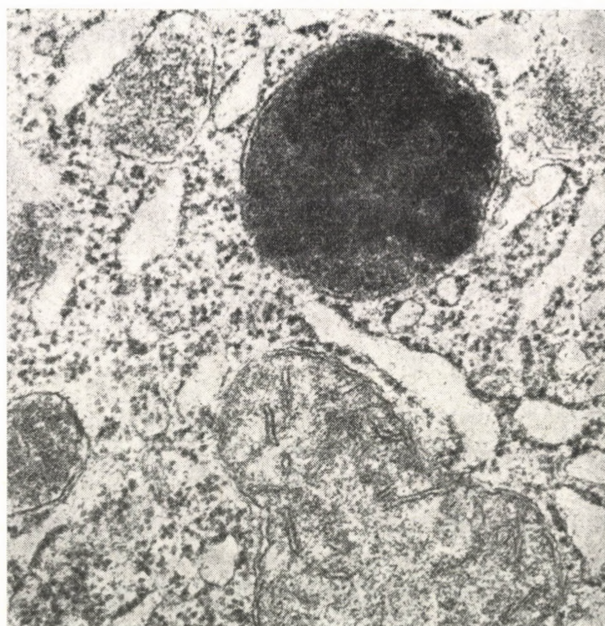

Fig. 9. Secondary lysosome containing iron in the liver parenchyma of a 5-day-old piglet following Chinofer treatment. EM $\times 65,000$

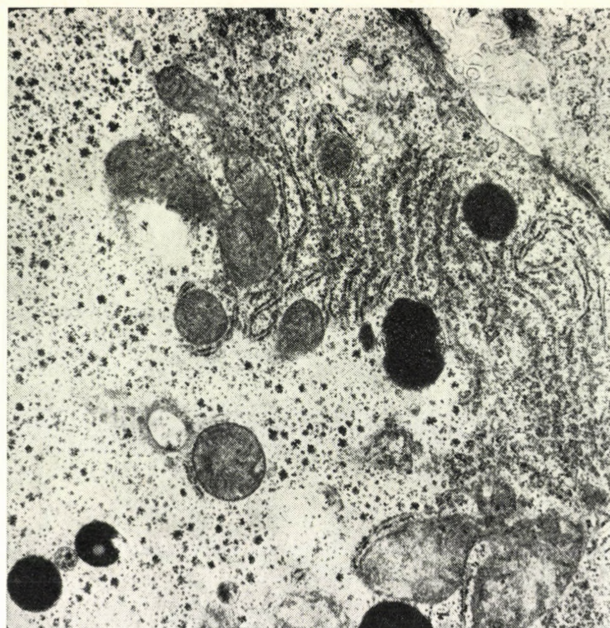

Fig. 10. **Iron-containing** secondary lysosome occasionally occurring in the liver parenchyma of a 5-day-old piglet treated with ferrous oxalate. EM ×22,000

Table I

Iron concentration and total iron-binding capacity of the blood plasma in suckling **pigs** before iron treatment at 3 or 19 days of age and 48 h thereafter (in mmol/l)

	Age			
	3 days		5 days	
	Iron concentration	Total iron-binding capacity	Iron concentration	Total iron-binding capacity
Control	19.74	65.99	7.33	108.30
Ferrous oxalate through a stomach tube	12.22	76.15	13.16	83.48
Chinofer inj. ad us. vet. im.	14.29	56.97	42.30	99.83
Leodex inj. ad us. vet. im.	15.42	95.89	171.09	597.88
Master-mix inj. ad us. vet. im.	17.79	102.66	181.06	439.39
	19 days		21 days	
Control	7.62	144.39	3.57	154.35
Ferrous oxalate through a stomach tube	8.56	132.27	19.74	139.32
Chinofer inj. ad us. vet. im.	9.87	123.24	73.33	156.24
Leodex inj. ad us. vet. im.	8.27	142.70	183.13	597.88
Master-mix inj. ad us. vet. im.	7.71	144.11	189.80	597.88

Dose: 100 mg Fe/kg body weight

other digested material, and occasionally in vacuoles containing dense material and differing in structure from the former type (Fig. 9).

After treatment with ferrous oxalate, iron was demonstrated in the liver cells only occasionally and in negligible quantities. The endothelial and Kupffer cells of these animals did not differ from those of the untreated controls. Dense vacuoles containing iron were observed in some places in the parenchyma (Fig. 10).

In addition to morphological studies on the absorption and tissue distribution of iron, the iron concentration and total iron-binding capacity of the blood plasma were also determined, using paired blood samples withdrawn prior to iron treatment and prior to slaughter. Individual values obtained for the 10 animals at different blood sampling times are shown in Table I.

Discussion

The present studies have shown differences between the absorption of the examined preparations and their distribution in the organism. Some preparations (Leodex and Master-mix inj.) were absorbed rapidly and efficiently from the injection site and distributed uniformly in the organism within a short time, thus rendering possible the filling-up of physiological iron stores. The administration of these preparations was accompanied by an appearance of high iron concentrations in the blood plasma and a simultaneous increase of the total iron-binding capacity. In this case the iron-binding proteins may become saturated or even over-saturated. The health hazards of over-saturation (iron toxicosis, temporary inhibition of the macrophage system of the organism, etc.) are well-known (Süveges and Glávits, 1976; Glávits and Süveges, 1977).

Chinofer was absorbed slowly and persisted at the injection site for a longer time, thus forming a "local iron store" from which iron was transported to the sites of use during a prolonged time but in sufficient quantities. In this case the above-mentioned side effects are less probable to occur. However, around the injection site the discolouration of tissues in smaller or larger areas and the development of other local reactions (degeneration of muscle fibres, inflammation, abscess formation) must be also reckoned with.

After administration of Hemogen through a stomach tube, iron concentration of the blood was satisfactory. The same conclusion was drawn by Misley et al. (1978) and Misley and Sárközy (1980). Iron and dextran were observed together in different organs and cells following the administration of any of the three injection preparations.

The present findings have led us to the conclusion that the effects and other afore-mentioned characteristics of the four iron preparations were not

influenced appreciably by the age of the treated animals. The effects of these preparations on the organism bear a relation to the mode of their administration and their qualitative properties not examined by us.

Acknowledgement

The authors are indebted to Dr. Attila Misley, Assistant Lecturer at the Department of Pharmacology, University of Veterinary Science, Budapest, for performing the blood analyses and for providing their results.

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PELVIMETRIC DIFFERENCES BETWEEN TWO GENERATIONS OF A BEEF CROSSING A PRELIMINARY STUDY

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A comparison between 59 F_1 and 8 R_1 females from the crossing between the beef-purpose Limousine cattle and the local Hungarian Fleckvieh type, dual-purpose cattle revealed significant differences only between the variances of internal pelvic measurements. In spite of the expected genetical regression following probable heterosis in the F_1 generation, such an effect could not be detected. Student's *t* test did not permit the rejection of the null hypothesis in terms of mean values. Variances, on the other hand, seemed to be significantly greater in the R_1 generation, except for the median diameter of the pelvis. This shows the expected greater homogeneity of the F_1 generation.

Keywords. Beef crossing, generations, pelvimetric differences, pelvic measurements, statistical analysis.

Reproduction is a fundamental aspect of animal husbandry. This holds particularly true when the offspring represents the main output. Calving difficulties are thus directly related to the efficiency of beef farming. The anatomy of the birth canal in cows is one of the least favourable among domestic mammals because of the narrow, cranially bulging shape and the high lateral edges of the ischiadic bone (Kovács, 1958). Certain crossing programmes including breeds of various sizes and growth intensities may need surgical assistance at calving, which decreases profitability even if the intervention is successful (Menissier, 1974).

Various generations of the crossings between Hungarian Fleckvieh and Limousine cattle have been subjected to intensive study recently as the proportion of these animals increased in the specialized beef production of Hungary (Csomós, 1981).

In spite of favourable calving properties, pelvimetric investigations were carried out in the first two generations of the crossings. The evaluation of the data may help a better understanding of the birth canal in relation to genetic changes.

Materials and methods

The following internal pelvic measurements were taken in vivo on 59 F_1 and 8 R_1 (75% Limousine) females at the Nagykörös State Farm:

1. *Median diameter* — conjugata vera: measured between the promontory of the sacral bone and the cranial point of the symphysis.

2. *Interpsoadic distance* — diameter transversa aperturae pelvis intermediae: measured between the psoadic tubercles.

3. *Intertuberous distance* — diameter transversa aperturae pelvis caudalis: measured between the ischiadic tubers.

In addition to these standard measurements (Kovács, 1967; Fehér, 1980) a two-dimensional, synthetic value was calculated (Holló et al., 1976):

4. *Area of the ellipse* defined by the median diameter and the interpsoadic distance.

This latter variable was introduced to estimate the degree of spaciousness inside the birth canal at a critical segment.

Due to technical difficulties, internal pelvic measurements are difficult to gather from female calves or even young heifers. For this reason, individuals were studied only around the time of first artificial insemination. Although the aim of this paper is a comparison between Limousine crosses from this relatively young age group, it may be of interest here that the incidence of complicated calvings (measured by the percentage of Caesarean sections and major complications) shows a rapid decline with age as observed in the Charolais breed (Menissier, 1974): as the area of the pelvic aperture increases from around 190—300 cm^2 in two years old heifers up to 385—425 cm^2 in cows beyond the fourth year, calving difficulties decrease from 15—30 to 0—5%.

The above-listed anatomical characteristics provided the input data for the statistical analysis which was used to test the following hypotheses:

H_0: On the basis of this sample, no difference can be observed in the mean values and standard deviations between the F_1 and R_1 generations.

H_r: There is a significant difference, at least in the case of certain measurements, at the $P \leq 0.05$ level, and this provides a basis for an at least 60% correct quantitative classification.

This pair of hypotheses was tested both in terms of mean values and variances. Student's *t* test and a discriminant analysis (using F tests of variance in a stepwise manner) were carried out to study the problem.

Results

Univariate statistics on the two groups of offspring and the results of the *t* test are summarized in Table I.

Due to the lack of significant differences between the mean values, another approach was necessary for detecting possible differences. The data were subjected to a stepwise discriminant analysis, which selected intertuberous distance as the variable of most distinctive value on the basis of variances. Results of this procedure are presented in Table II.

Discussion

The recognition of intertuberous distance as the dimension with most discriminating value between the two groups of offspring is the first interesting phenomenon. Rump width measured between the pin bones is the external measurement analogous to this pelvic dimension. It seems to be diagnostic of the developmental stages of the skeletal system during cattle ontogeny (Bartosiewicz and Gere, 1981). The connection between the caudal region of the pelvis and the overall bone structure requires further investigations.

Table I

Univariate tests of differences between the mean values (*t* test) and variances (squared standard deviations; F test) of the two generations of Red and White Hungarian and Limousine crosses

Grouping variables	Generations			Differences	
	F_1	R_1	Both groups	Values t	P level (LT)
number of cases	59	8	67		
Mean values (cm)					
Median diameter	17.338	18.062	17.425	0.025	—
Interpsoadic distance	13.217	13.937	13.302	0.025	—
Intertuberous distance	13.338	14.375	13.506	0.029	—
Ellipse (cm^2)	180.776	198.625	182.907	0.029	—
Standard deviations (cm)				F	
Median diameter	0.993	0.623	0.960	3.999	0.05
Interpsoadic distance	0.787	0.863	0.796	5.773	0.01
Intertuberous distance	0.792	1.157	0.839	9.746	0.001
Ellipse (cm^2)	15.749	19.023	16.133	8.622	0.001
Coefficients of variation (%)					
Median diameter	5.728	3.450	5.511		
Interpsoadic distance	5.958	6.195	5.983		
Intertuberous distance	5.916	8.051	6.212		
Ellipse	8.712	9.577	8.821		

Table II

Results of the discriminant analysis

Variable entered:	Summary of discrimination parameters		
	F value to enter:	U statistic:	Degrees of freedom:
Intertuberous distance	9.7463	0.8696	1 65

Generations	Classification matrix			
	Generations		Total expected	Percentage correct
	F_1	R_1		
F_1	40	19	59	67.8
F_2	3	5	8	62.5
Total obtained	43	24	67	67.2

The insignificant differences between mean values of pelvic measurements in the two generations under discussion do not even fall in line with the tendency of genetic regression, that is, the expected slight decrease of dimensions relative to the F_1 generation. The virtually greater averages obtained for the second generation of the crossing even suggest some improvement brought about by the Limousine breed in this respect.

Due to significant differences between the variances of the two groups (Table I), the percentage of correct classifications exceeded 60% (Table II). In addition to the intertuberous distance, the high F value of the synthetic variable means that variances of all three pelvic dimensions are different between the two groups. Except for the median diameter, homogeneity decreased.

The relationships between the pelvic measurements, grouping variables (coded versions of the concepts of the F_1 and R_1 groups) and sample size are illustrated by the histogram of the canonical variable in Fig. 1. The distribution of cases is the graphic representation of the classification matrix shown in Table II.

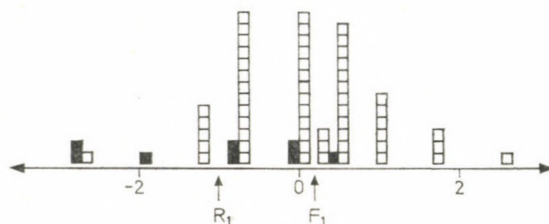

Fig. 1. Distribution of individuals along the canonical variable symbolizing the two grouping variables (generations). Letters in the histogram stand for group averages. Black units represent R_1 (75% Limousine) individuals

Acknowledgements

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BOOK REVIEWS

KASSAI, Tibor: Handbook of *Nippostrongylus brasiliensis* (Nematode). Commonwealth Agricultural Bureaux, Farnham Royal, Slough, U.K. and Akadémiai Kiadó, Budapest, Hungary, 1982. 257 pages, 51 figures (partly colour), 12 tables.

Nippostrongylus brasiliensis, a small nematode parasite which inhabits the intestine of the rat and mouse, has been widely recognized as an almost uniquely useful model for studies in general parasitology, immunology, allergology, experimental chemotherapy and many other fields of biomedical sciences. The resulting literature numbers more than 700 scientific publications rendering extremely difficult in searching the voluminous and scattered information on this parasite. Having been working with this model for 15 years, the author attempts in this volume to summarize the fundamental characteristics of the model and to guide the Reader through the abundant data published on it.

The novel layout of the book has been devised to provide maximum information and flexibility within a minimum space.

The book is divided into three main parts. The first section is an illustrated account of the basic characteristics of the model, which includes the taxonomy, morphology, life-cycle, physiology, methods of culture and handling of the parasite, the immunological phenomena and pathological processes induced by this nematode in the host, and the application of the model for screening drugs for anthelmintic activity. The chapter dealing with the immunological consequences of infection is of special significance because the model had provided the experimental base for much of our understanding of the immunopathology of nematode infections. Keywords in the margin facilitate orientation.

The second part consists of an up-to-date list of references (737 entries), each being followed by a digest of the information contained in the reference, including the purpose of study, methods used, and results obtained. Digests do not conform to the traditional pattern of abstracting journals and annotated bibliographies. By applying a novel format in this section, the selection of meaningful references according to the user's needs will be greatly facilitated.

The third section is made up of an author index and a particularly detailed subject index (pp. 44). The aim has been to select and arrange entries in a way that makes this section both stimulating and informative.

Finally, a list of institutions where *N. brasiliensis* is maintained is included.

The handbook provides a comprehensive introduction to the parasite, and a guidance to the related literature. Both research students entering the field, and more experienced research workers will find this book as an invaluable aid in exploiting the potential usefulness of this model of biomedical research.

F. HOLLÓ

Hygienic Problems of Animal Manures. Proceedings of a joint workshop of expert groups of the Commission of the European Communities, German Veterinary Society (DVG) and Food and Agricultural Organization, Ed. by D. Strauch, University of Hohenheim, Stuttgart, Federal Republic of Germany, 1983. pp. 314.

The Proceedings of a joint workshop of expert groups of the Commission of the European Communities, the FAO-European Network on Animal Waste Utilization — Subnetwork 1

and the Workshop of Animal Hygiene of the German Veterinary Society (DVG) contain papers read and proposals offered at the joint meeting held at Hohenheim-Stuttgart, Federal Republic of Germany between October 11 and 13, 1982, with 52 participants.

The Proceedings include papers belonging to 5 themes, together with conclusions and the minutes of the joint meeting of the three expert groups.

Papers of *Theme 1* deal with hygienic aspects of storage, handling and composting of manures, and the survival times of manure-borne pathogenic microorganisms are reported. The introductory paper outlines liquid manure disinfection methods, known survival times of microorganisms in the environment and in liquid manure, and proposes a liquid manure utilization system suitable for reducing potential hazards constituted by liquid manure disposal to the minimum. The remaining papers deal with the evaluation of indicator bacterial counts, survival of salmonellae during composting solid animal manure, and hygienic consequences of manure handling inside the stable. Several practically applicable suggestions and conclusions are also presented, among others about the practical application of knowledge of the expectable survival time of salmonellae in liquid manures stored under different conditions.

Papers of *Theme 2* discuss hygienic aspects of chemical disinfection of manures. For the inactivation of viruses, lime has proved to be the agent of choice under field conditions. Conditions under which salmonellae or Aujeszky's disease virus can be inactivated with lime, and salmonellae with peracetic acid, are also described.

To prevent environmental contamination brought about by chemical disinfectants, liquid manures must be processed in a way facilitating efficient disinfection with lower doses of disinfectants, as it is established in the comprehensive paper of Theme 2.

Theme 3 includes papers examining the economic and hygienic aspects of anaerobic or aerobic, mesophilic or thermophilic treatment of manures. A comparative evaluation has shown that of the ways of using liquid manure as an energy source, lowest energy costs can be achieved in the case of aeration with direct heat recovery. For the time being, biogas production in the systems listed is uneconomical. However, more economical utilization of heating pumps can be expected in the future.

Several papers of Theme 3 deal with the effect of aerobic-thermophilic treatment of liquid manure on viruses. It has been found that foot-and-mouth disease virus, Aujeszky's disease virus and SVD virus are inactivated within 25 to 45, 30 to 48 and 28 to 45 hours, respectively. After mesophilic treatment, the survival time of Talfan virus and Coxsackie virus is 2 to 7 weeks. During biogas production, staphylococci and salmonellae are killed within 2 weeks. At the temperature of thermophilic biogas production (55 °C), both salmonellae and *Ascaris* eggs are inactivated within 24 hours. Several authors report the effect of feed additives, antibiotics and disinfectants on biogas production.

Theme 4 includes papers on legal and practical approaches for management and disinfection of infected manures in some countries, with particular reference to Ireland, the Netherlands and the Federal Republic of Germany.

Theme 5 contains papers giving information on the technical visits. Treatment and processing of liquid manure at the experimental station of Hohenheim University and biogas production at a biogas plant visited by the participants of the meeting are described.

At the meeting, the activity of the FAO-European Network "Animal Waste Utilization", and especially that of its Subnetwork 1, was evaluated by Head of Subnetwork 1, Professor I. Ekesbo (Sweden). He summarized the achievements of the co-operation and outlined future tasks. Subsequently the participants discussed a draft report concerning the hygienic aspects of liquid manure handling, intended for presentation at a FAO meeting.

In conclusion, it can be established that the thought-provoking papers read at the meeting and the spirited and fruitful discussion following them have facilitated progress in this field.

Between April 19 and 22, 1983, Food and Agricultural Organization — European Network on "Animal Waste Utilization" held a meeting in Budapest, Hungary. At this meeting, members of Subnetwork 1 evaluated comments on the draft report presented at the Hohenheim meeting, and Dr. Géza Tamási (Hungary) was commissioned to compile the report concerning the hygienic aspects of liquid manure handling in its final form. This final report will be discussed and accepted at the next meeting of FAO Subnetwork 1 in Brno (Czechoslovakia) in 1984.

G. TAMÁSI

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STUDIES ON THE PROTEASE INHIBITOR OF *KLEBSIELLA AEROGENES*

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(Received June 1, 1983)

A protease inhibitor was prepared and purified by affinity chromatography from cell extracts of *Klebsiella aerogenes*. The purified material was found to inhibit the activity of both trypsin and chymotrypsin. The inhibition of both enzymes depended on the same active site. The molecular mass of the inhibitor was between 28,000 and 30,000.

Keywords. *Klebsiella aerogenes*, protease inhibitor, purification.

In a previous paper, we have reported the recovery of a protease inhibitor from cell extracts of *Proteus vulgaris* by purification, using affinity chromatography. The inhibitor was active against both trypsin and chymotrypsin, and the trypsin- and chymotrypsin-inhibiting activity was associated with the same molecule. It has been proved by enzymatic pretreatment that inhibition can be attributed to the same, or to closely adjacent, active sites. The molecular mass of the inhibitor was in the range of 30,000 to 32,000 as determined by gel chromatography (Juhász, 1982).

Recently, using similar methods, we examined the protease inhibitor of *Klebsiella aerogenes*, an inhibitor the existence of which was first reported by Fossum (1970). Our results are reported in the present paper.

Materials and methods

Two *K. aerogenes* strains isolated from pigs were used. The methods were described in detail by Juhász (1982).

Results

The crude bacterial extracts showed neither trypsin nor chymotrypsin activity. The inhibitory activities are shown in Table I.

Aliquots of both extracts were purified by affinity chromatography on a Sepharose–chymotrypsin column (Table II).

Both inhibitors bound to the gel, and were desorbed upon elution with HCl with an efficiency of about 70%. The purified inhibitor solution was

treated with chymotrypsin and tosyl-phenylalanine chloromethyl ketone trypsin (TPCK trypsin) (Table III). Both chymotrypsin and trypsin pretreatment resulted in the disappearance of the inhibitory activity exerted on the homo-

Table I

Inhibitory activities of crude extract and bacterial cell mass of *K. aerogenes*

	Bacterial cell mass g	Volume ml	Trypsin inhibitor		Chymotrypsin inhibitor	
			U/ml	U/g of bacterial cell mass	U/ml	U/g of bacterial cell mass
Extract 1	0.125	6.25	52	2600	94	4700
Extract 2	0.170	8.50	68	3400	104	5200

Table II

Recovery of the trypsin and chymotrypsin inhibitor from extracts of *K. aerogenes* by affinity chromatography

	Inhibitory activity (U) for			
	trypsin		chymotrypsin	
	extract 1	extract 2	extract 1	extract 2
Crude extract (6 ml)	312	408	564	624
Fractions eluted by borate buffer (40 ml)	0	0	0	0
Fractions eluted by HCl (4 ml)	210	269	435	440
Recovery (%)	67.3	72.5	77.1	70.5

Table III

Effect of pretreatment with homologous enzymes on the purified inhibitor of *K. aerogenes*

	Inhibitory activity (U) for		Enzyme activity (U)	
	trypsin	chymotrypsin	trypsin	chymotrypsin
Inhibitor, before treatment (0.2 ml)	10.5	14.9	0	0
Chymotrypsin (0.6 ml)	0	0	0	900
Inhibitor, after pretreatment with chymotrypsin	0	n.s.	n.s.	n.s.
TPCK-trypsin (1.0 ml)	0	0	260	0
Inhibitor, after pretreatment with TPCK-trypsin	n.s.	0	n.s.	n.s.

n.s. = not studied
The tests were performed in triplicates

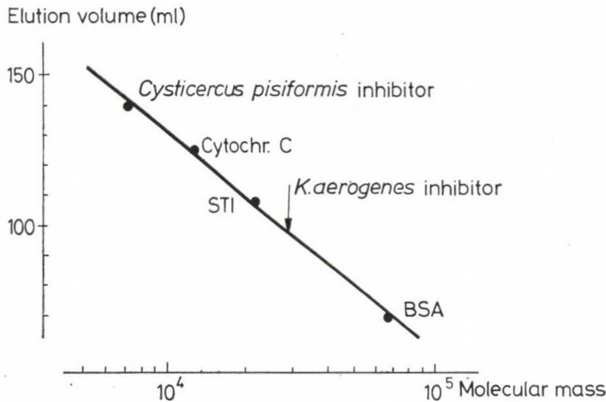

Fig. 1. Relationship between elution volume and molecular mass on a Sephadex G-75 column $\log M_r = 5.8084—1.5902\ K_{av}\ (r^2 = 0.9989)$

logous enzyme, indicating that the same (or very closely adjacent) active sites were responsible for inhibition of the two enzymes.

The molecular mass of the inhibitor was found to be in the range of 28,000 to 30,000, as determined by gel chromatography (Fig. 1).

Discussion

The properties of the inhibitor studied in the present work closely resemble those of the protease inhibitor of *P. vulgaris* (Juhász, 1982).

The protease inhibitors of both bacteria act on both trypsin and chymotrypsin, i.e. their active sites are able to inhibit both of these enzymes. The molecular mass of the protease inhibitors of the two bacteria is practically identical.

These characteristics, although they cannot be considered general for the Enterobacteriaceae family, allow us to draw the conclusion that the occurrence of such protease inhibitors active against trypsin and chymotrypsin and of relatively large molecular mass (30,000) can be reckoned with within this family.

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Acta Veterinaria Hungarica 31 (4), pp. 125—129 (1983)

STUDIES ON THE ROLE OF PROTEASE INHIBITORS IN THE PATHOGENESIS OF SWINE DIARRHOEA CAUSED BY *ESCHERICHIA COLI*

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(Received June 1, 1983)

Haemolytic and non-haemolytic *E. coli* strains isolated from swine, and strains of other genera of the Enterobacteriaceae family *(Klebsiella aerogenes, Pseudomonas aeruginosa, Proteus vulgaris, Salmonella typhimurium, Salmonella derby, Shigella flexneri, Shigella sonei)*, were examined for trypsin- and chymotrypsin-inhibitors. No extracellular inhibitors were detected. No correlation seemed to exist between inhibitory activity and enteropathogenicity. The bacterial protease inhibitors, being of low activity, cannot account for the striking decrease in protease activity observed in diarrhoeic swine.

Keywords. Protease inhibitors, pig, diarrhoea, *Escherichia coli.*

The literature is poor in data on bacterial protease inhibitors. Høyem and Skulberg (1962) reported the presence of a trypsin-inhibiting factor in culture supernatants of *Clostridium botulinum.* Brecher and Pugatch (1969) detected a trypsin and a chymotrypsin inhibitor in extracts of *E. coli.* Fossum (1970) found trypsin- and chymotrypsin-inhibiting activity in cells of Gram-negative bacteria *(Proteus mirabilis, Proteus vulgaris, E. coli, Klebsiella* sp., *Serratia marcescens* and *Pseudomonas aeruginosa).* Recently the protease inhibitor of *P. vulgaris* and *K. aerogenes* has been studied for molecular mass and active site (Juhász, 1982, 1983).

The activity of intestinal proteases is significantly reduced in pigs affected by *E. coli* diarrhoea (Mészáros et al., 1967, Juhász et al., 1967). The question has arisen whether protease inhibitors of the growing bacterial mass contribute to this reduction. Therefore, (i) we compared the inhibitor content of pathogenic and non-pathogenic bacterial strains and (ii) examined whether intestinal proteases have an enzymatic effect on intestinal bacteria.

Materials and methods

Table I shows the strains studied.

Bacterial cultures were plated on nutrient agar in Petri dishes and incubated at 37 °C for 2 days. Subsequently, bacterial growth on the agar surface was scraped off. To release intracellular inhibitors, the cells were washed 3 times in saline, centrifuged (19,000 *g*, 10 min), suspended in distilled

Table I

E. coli strains enteropathogenic and non-enteropathogenic for swine and other strains of different genera of Enterobacteriaceae

Strain	Number of strains studied
E. coli 0138:K81 haemolytic (a,b)	2
E. coli 0139;K82 haemolytic (a,b)	2
E. coli 0141;K85,88 haemolytic (a,b)	2
E. coli 0149;K91,88 haemolytic (a,b)	2
E. coli 017;K16 non-haemolytic	1
E. coli 018;K76 non-haemolytic	1
E. coli 0113;K75 non-haemolytic	1
E. coli 0128;K67 non-haemolytic	1
Klebsiella aerogenes (a,b)	2
Pseudomonas aeruginosa (a,b)	2
Proteus vulgaris (a,b)	2
Salmonella typhimurium (a,b)	2
Salmonella derby	1
Shigella sonei	1
Shigella flexneri	1

water and sonicated in an MSE sonifier for 40 min (1.2 A). Further release of inhibitors from the cells was facilitated by boiling for 5 min followed by centrifugation for 40 min (19,000 g). The supernatant was tested for inhibitory activity and it was freeze-stored until used (Fossum, 1970).

Enzyme activities were determined spectrophotometrically, in TRIS buffer, pH 7.8, in a volume of 3 ml. The trypsin activity was determined by the method of Schwert and Takenaka (1955), using N-α-benzoyl-L-arginine ethyl ester as substrate, by measuring the extinction at 253 nm; the chymotrypsin activity determination was performed by the method of Hummel (1959) with an N-benzoyl-L-tyrosine ethyl ester substrate, by measuring extinction at 256 nm, after a pre-incubation period of 2 min, which is needed for enzyme-inhibitor binding.

One unit (U) of enzyme activity is defined as the amount of enzyme hydrolysing 1 μmol substrate per min, at 25 °C. One unit of inhibitory activity is defined as the amount of inhibitor which inactivates one unit of enzyme.

An inhibitor-active *K. aerogenes* strain and an inactive *E. coli* 017;K16 strain were suspended in a saline solution containing 2 mg/ml trypsin and 0.1 mg/ml chymotrypsin. The trypsin and chymotrypsin activities of the system were 2100 U/ml and 3150 U/ml, respectively. (For comparison: in the normally-functioning small intestine of swine the trypsin and chymotrypsin activities

are 1000—10,000 U/ml and 2000—10,000 U/ml, respectively). The same test strains were suspended in enzyme-free saline to serve as controls. The initial bacterial counts corresponded to 10^3 colony-forming units (cfu) per ml. The bacterial suspensions were incubated at 37 °C for 24 h, whereafter the cfu values were determined again.

Results

In the supernate obtained after the first washing in saline and the subsequent centrifugation of the cell suspensions, neither protease activity nor protease inhibitors could be detected. The intracellular extracts, measured

Table II

Intracellular trypsin and chymotrypsin inhibitor activities of certain bacterial strains

	Inhibitor (U/g wet weight)	
	Trypsin	Chymotrypsin
E. coli 0139;K82 (a) haemolytic	200	200
E. coli 0149;K91,88 (b) haemolytic	200	200
Pseudomonas aeruginosa	200	200
Klebsiella aerogenes (a)	2600	4700
Klebsiella aerogenes (b)	3400	5200
Proteus vulgaris (a)	6500	8800
Proteus vulgaris (b)	7100	10400

after sonication but before boiling, showed no measurable protease activity but did have inhibitory activity. Inhibitory activity was higher after boiling.

Among the *E. coli* strains studied, only two strains (0139;K82, *a* and 0149;K91,88, *b*), both haemolytic and both isolated from swine showing symptoms of enteric disease, were found to possess intracellular inhibitory activity, even these at very low levels (200 U/g of wet weight of bacteria, for both inhibitors). None of the non-enteropathogenic strains isolated from healthy swine contained inhibitors.

Among the other strains, both of the *P. vulgaris* and both of the *K. aerogenes* strains possessed an easily-detectable inhibitory activity while the activity of one of the *Pseudomonas aeruginosa* strains was low (Table II).

Neither the inhibitor-active *K. aerogenes* nor the inactive *E. coli* test strain multiplied in the enzyme-free saline while both multiplied in the enzyme-containing one. In the latter medium the initial 10^3 cfu/ml value rose to 10^8—10^9 cfu/ml within 24 h with both strains.

Discussion

Although protease inhibitors of Gram-negative bacteria were first described more than 10 years ago, no attention has been paid to them since. It would be important to clarify their biological role since inhibitor-positive Gram-negative bacteria play a considerable part in gastroenteritis and it is not impossible that their inhibitors influence the pathomechanism of these diseases in some way. Therefore, it seemed to be reasonable to seek answers to the two questions mentioned in the Introduction.

First, we wished to see whether bacterial inhibitors had any effect on the enzymes functioning in the host's intestine. The absence of extracellular inhibitors spoke against such an assumption. However, intracellular inhibitors released by bacteria disintegrating in the gut may get in contact with the host's proteases. One g of wet weight of ***P. vulgaris*** (the strain showing the most pronounced inhibitory activity in our experiments) had an inhibitory activity of 6500 and 7100 U for trypsin and that of 8800 and 10,400 U for chymotrypsin. (Naturally, we only have data on strains grown on nutrient media, and these may not reflect the events in the intestines.) One ml of small-intestinal contents from healthy swine showed inhibitory activities of 1000—10,000 U and 2000—10,000 U for trypsin and chymotrypsin, respectively. Taking into account the enzyme- and inhibitory activities, we are sure that it is impossible that even large quantities of bacteria could cause the drastic decrease of enzyme activity observed in enteritis. On the other hand, it is possible that bacteria may cause a less expressed decrease in enzyme activities of small-intestinal proteases.

Bacteria were found to possess only intracellular (and no extracellular) protease inhibitors. It seems therefore probable that the primary function of inhibitors is not to protect bacteria against exogenous enzymes; more probably, they participate in the regulation of the bacterial enzyme system. Our results have not revealed the exact biological role of the inhibitors.

Although some of the *E. coli* strains tested by us possessed intracellular inhibitory activity, this was not characteristic of strains of enteropathogenic antigenic structure and of haemolytic ability. Therefore, we assume no correlation between inhibitory activity and pathogenicity.

The second question was whether the protease activity of small-intestinal contents influences the multiplication of bacteria, and whether inhibitors can protect bacteria against proteases. From in vitro experiments it was concluded that in the presence of proteases, strains lacking inhibitors multiplied at least as well as those containing inhibitors. Furthermore, in a medium containing no other source of energy, the multiplication of both inhibitor-active and inactive bacteria was advantageously influenced by the presence of the enzymes. Presumably, the bacteria utilized the enzymes as energy (and protein)

sources. Therefore, under the present conditions of experiment, protease inhibitors did not influence bacterial multiplication in the presence of protease enzymes.

Our results do not agree in all detail with those reported by Fossum (1970). Thus, although we succeeded in demonstrating inhibitory activity in a few *E. coli* strains, this activity was very weak. The same applies to *Pseudomonas aeruginosa*. Similarly to Fossum (1970), we detected inhibitors in *K. aerogenes* and *P. vulgaris* strains.

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STUDY OF THE DURATION OF IMMUNITY OF SHEEP VACCINATED AGAINST SHEEP POX WITH A LIVE VIRUS VACCINE

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The duration of immunity to one dose of 1000 $TCID_{50}$ live virus vaccine prepared from the "Perego-M" sheep pox virus strain propagated in cell culture was studied. Vaccinees proved to be fully protected for at least 18 months post vaccination against a challenge infection with 200 ID_{50} of virulent virus. Only one out of 4 animals was protected 24 months, and none 36 months, post vaccination. Yearly revaccination of the whole flocks at a season when their lambs are 2 to 3 months old keeps them under continuous solid protection. In the last three years 5 million doses of this vaccine were applied in the endangered area and since that time no case of sheep pox has occurred.

Keywords. Sheep pox, live virus vaccine, immunity.

Freeze-dried vaccine produced from sheep pox virus adapted to cell culture has been used in Mongolia since 1979 (Perenlei and Sólyom, in press; Sólyom et al., 1980). To develop a practical vaccination programme, the persistence of immunity due to vaccination had to be determined.

Several publications have appeared on live virus vaccines adapted to different cell cultures (Adlakha et al., 1971; Aygün, 1955; Köylü and Nada, 1971; Lang and Leftheriotis, 1961; Martin et al., 1973; Mateva-Pankova et al., 1974; Ramyar, 1965; Ramyar and Hessami, 1970). However, scanty information is available about the duration of immunity produced by such vaccines.

The potency of vaccines has been tested by two methods. Either the vaccinees were challenged with a fix dose (50 to 1000 ID_{50}) of virus, or the infective virus was titrated parallel on the skin of vaccinees and of unvaccinated animals (so-called "index of protection" method). Ramisse et al. (1978) established that vaccinees were resistant for 3 months against 1 million ID_{50} of virus but this resistance level decreased to 1000 ID_{50} between the 4th to 6th months post vaccination (p.v.). Precausta et al. (1978) determined the index of protection for vaccinees 6, 12 and 20 months following vaccination. They found index values of 4.2, 3.1 and 1.1 in the 6th, 12th and 20th post-vaccination months, respectively. The aim of our study was to test the duration of immunity evoked by our vaccine, by means of challenge infection. Simultaneously, the potency of the vaccine was evaluated in the field.

Materials and methods

Vaccine

The "Perego-M" virus strain adapted to lamb testicle cell culture was used. The dose of the vaccine was 1000 $TCID_{50}$ virus in 1 ml. The vaccine was injected subcutaneously behind the shoulder.

Experimental animals

Twelve months old sheep of both the Mongolian and the Orhon breeds were used. The animals originated from an area where no sheep pox had occurred in the preceding 5 years. Both experimental groups contained sheep of the two breeds in equal proportion. 2×15 sheep were vaccinated, 15 served as control. During the observation period of 36 months, 15 animals were discarded in consequence of intercurrent diseases, thus, finally 18 vaccinated and 11 control sheep were challenged.

Challenge infection

The vaccinated sheep were challenged with a virus strain isolated from a natural outbreak of sheep pox in Mongolia (Stavrogaly). Both vaccinated and control sheep were inoculated with 200 ID_{50} of the virulent virus under the skin of the tail. The challenged animals were observed daily for 15 days for local reaction and fever.

Results

Duration of evoked immunity

In want of any information about the duration of immunity by this vaccine, yearly revaccination of whole flocks was carried out. To study the duration of postvaccination immunity, each of 15 sheep of the Mongolian and

Table I

Study of the duration of immunity in sheep vaccinated with live virus vaccine against sheep pox

	No. of protected		No. of challenged animals	
Months	12	18	24	36
Vaccinees	4/4*	6/6	1/4	0/4
Controls	0/3	0/2	0/3	0/3

* challenge dose: 200 ID_{50} virus per animal

Orhon breeds were vaccinated. These sheep were kept together, while 15 unvaccinated control sheep of the same breeds were kept separately. The sheep were vaccinated subcutaneously by one dose. Then 4 to 6 vaccinees and 2 or 3 controls were challenged 12, 18, 24 and 36 months p.v. The challenged sheep were observed for 15 days.

The results of the challenge tests are summarized in Table I. All challenged animals were protected up to the 18th month p.v., however, 25 months p.v. only one of 4 animals and 36 months p.v. none of the animals was protected against a challenge dose of 200 ID_{50} virus.

Discussion

The experiment proved that all sheep vaccinated with a dose of 1 ml (1000 $TCID_{50}$) were protected for one and a half year against a challenge infection of 200 ID_{50} virulent virus. These results are in agreement with those of Precausta et al. (1978), who checked the immunity of vaccinated sheep by means of the so-called "index method". The animals vaccinated by them resisted a challenge infection with 1250 ID_{50} virus 12 months p.v., however, 20 months p.v. they were resistant only against 12 ID_{50} challenge virus. Ramisse et al. (1978) used the same index method to test the immunity of sheep vaccinated with a live virus vaccine. They established that 6 months p.v. the vaccinees resisted a challenge with 10,000 ID_{50} virulent virus.

In view of the above results and the fact that maternal antibodies transferred via colostrum from immune ewes to their lambs would hinder the development of lasting immunity for the first 2 to 3 months of their life (Precausta et al., 1978), yearly vaccination of whole flocks in a season when the lambs reached this age, will result in full protection of them against sheep pox. The soundness of this vaccination schedule has been proved by the fact that no case of sheep pox occurred in that area of Mongolia where all sheep were regularly vaccinated for the last 3 years. In accordance with experiences obtained with an inactivated vaccine (Sólyom et al., 1982) no differences in the immune response to our live virus vaccine were observed in sheep of the two breeds tested.

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MASS ABORTION CAUSED BY INFECTIOUS RHINOTRACHEITIS (IBR/IPV) VIRUS IN A BEEF CATTLE HERD

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In the first quarter of 1982, 157 of 200 pregnant Hereford heifers purchased by a farm aborted between the 5th and 9th month of pregnancy.

Sixty-six fetuses and 3 placentae were examined. Histopathologically, and rarely also by gross examination, inflammatory-necrotic foci were found in the liver and spleen of more than half of the fetuses. Occasionally icterus was also observed. By microscopic examination and bacterial culturing, listeria, streptococci and staphylococci were demonstrated in one fetus each, and fungi in further 3 fetuses. All examined materials proved to be negative for brucella, leptospira and chlamydia. Infectious rhinotracheitis virus was isolated from 8 fetuses.

The majority of the aborted heifers were tested serologically and proved negative for brucellosis and leptospirosis. Some blood samples were tested also for chlamydial antibodies, all with negative results. The 50 blood samples tested for antibodies to infectious rhinotracheitis virus were positive, with titres 1 : 2–1 : 256 (geometric mean titre: 1 : 28).

In all probability, the stock consisting of the 200 purchased pregnant heifers seronegative for IBR/IPV must have contracted infection from the own seropositive stock of the farm. In the latter stock the rate of abortion was 5 to 10% under similar conditions.

In the present outbreak, high susceptibility of the stock, high exposure to infection due to the managemental technology and inappropriate hygienic conditions, and a marked impairment of the herd's natural resistance caused by unusually cold weather and faults in feeding must have jointly led to the severe abortion.

Keywords. IBR/IPV virus, abortion, beef cattle, first-calf heifer.

The genital form of infectious rhinotracheitis/infectious pustular vulvovaginitis (IBR/IPV) was first observed in the last century, and has been studied in great detail recently (Barenfus et al., 1963; Saxegard, 1970). The upper respiratory form of the disease was recognized in the United States in the mid-fifties, and the common aetiological agent of the two disease forms was identified in the late 'fifties (Madin et al., 1956; McKercher et al., 1959). These results were confirmed, among others, by Hungarian authors (Manninger et al., 1962; Manninger and Mészáros, 1975).

Subsequently also abortions were traced back to IBR/IPV virus infection, first in the United States following vaccination of pregnant cows with an attenuated live-virus vaccine. Soon afterwards, IBR virus was isolated from natural cases. Later on, similar observations were reported from other continents and several European countries (among others, by Chow et al., 1964; Crane, 1965; Flamini and Allegri, 1972; Higgins et al., 1981; Holter and Andrews, 1979; Kahrs and Smith, 1965; Kelling et al., 1973; Magnani et al., 1978; McKercher and Wada, 1964; Mehrotra et al., 1979; Owen et al., 1964; Reed et al., 1971; Sattar et al., 1965; Saxegard, 1970; Straub, 1966, 1978, 1982).

In the meantime, disease forms characterized by encephalitis and/or conjunctivitis also became known on several continents and in numerous countries (Abinanti and Plumer, 1961; Barenfus et al., 1963; Bartha et al., 1969; Dawson et al., 1962; French (1962*a*, 1962*b*); Hughes et al., 1964; Quin, 1961).

Pustular vulvovaginitis has long been known in Hungary. In the second half of the 'sixties, the occurrence of both the upper respiratory and the encephalitic forms was reported (Csontos and Maczkó, 1967; Bartha et al., 1969). Virological and virus-serological examinations performed in different parts of the country have proved a considerably wide occurrence and spreading tendency of the disease (among others, Mocsári et al., 1973; Kudron, 1979; Tanyi et al., 1979).

In Hungary, no abortion following natural infection by IBR virus has been reported so far.

The present paper describes an outbreak of mass abortion observed in Hungary. The outbreak, caused by the IBR virus, draws attention to some important implications.

Materials and methods

Gross pathological, histopathological, bacteriological and mycological examinations

After gross pathological examination, the fetuses were examined by routine diagnostic methods. Smears prepared from the placentae and fetal stomachs were stained according to Stamp's method.

Culturing of bacteria was attempted from placenta, stomach, liver and lung samples, while that of fungi from stomach samples. Common agar, blood agar and beer agar were used as nutrient media. The cultures were incubated at 37 °C, under aerobic conditions in an atmosphere containing 5–10% carbon dioxide for 7 days. The results were read daily.

For the demonstration of leptospires, liver and kidney samples collected from fetuses were fixed in 10% formalin and processed by Levaditi's silver impregnation technique.

Frozen sections were prepared from liver, spleen and kidney samples fixed in formalin, and were stained with haematoxylin and eosin.

Virological and virus-serological examinations

For virus isolation, pooled organ homogenates of the inner organs were used, or, in some cases, the liver, spleen, kidney, gastrointestinal tract and lung of the fetuses were processed separately, depending on the degree of

autolysis of fetuses. The homogenates were inoculated into primary calf testicle and primary and secondary calf kidney monolayer cell cultures. The cell cultures were read daily and three to six blind passages were made at intervals of 3 to 5 days.

The virus-serological examinations and the identification of the isolated strains were performed by methods used in routine diagnostic work in Hungary (Csontos and Maczkó, 1967; Bartha et al., 1969; Mocsári et al., 1973; Kudron, 1979; Tanyi et al., 1979).

Serological examinations

In brucella and leptospira examinations, blood samples were first tested by a micro method, and only positive sera or those giving doubtful results were retested by a macro method.

As antigens, *Brucella abortus* marketed by Phylaxia Veterinary Biologicals and Feedstuffs Co. (Budapest, Hungary) for use in the agglutination and complement fixation tests, and leptospira strains cultured in Korthoff's medium for 3–4 days were used. In the chlamydia complement fixation test an antigen designated "Odessa", produced in the USSR, was used.

Results

In the autumn of 1981, farm "Sz" purchased 200 first-calf heifers of the Hereford breed. The heifers were kept on a separate pasture, isolated from the Hereford and Limousine herds that had been kept on the farm for a longer time.

At the end of November, the purchased heifers were mixed with the 50 "own" first-calf heifers of the farm. With the onset of winter, the purchased (A) stock and another stock of similar size (B) were put to separate areas of a pasture, close to a detached farm. Feeding and watering of the two herds were performed in an isolated manner. The feed, consisting mainly of corn silage and hay, was spread on the ground for the animals. The winter was unusually severe, the water-pipes were frozen several times, leading to disturbances in water supply. In some cases, because of temporary disturbances in water supply, animals of the two herds had to be watered from the same drinking troughs, although not at the same times. In addition to the above two herds, farm "Sz" possessed further two beef herds (herds C and D). These were wintered in the neighbourhood of a more remote farmstead.

Due to the severe, long winter and the poor feeding and keeping conditions, the body condition of animals deteriorated. They were kept in the open field, unsheltered, without dry resting places. Their need for proper resting areas was well indicated by the fact that they lay down on the hay freshly

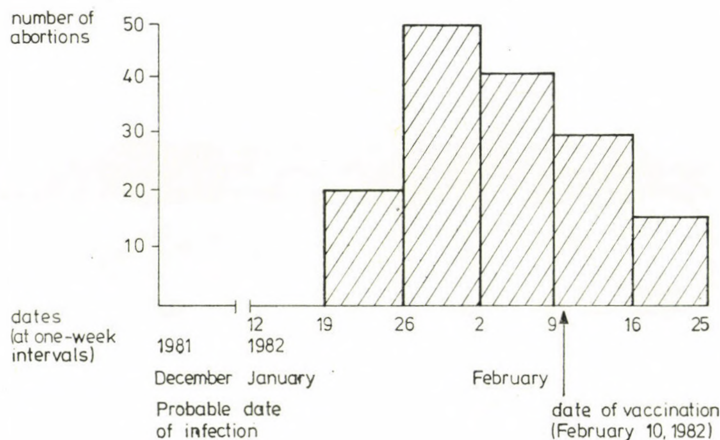

Fig. 1. Time-course of the abortions in the Hereford herd of 200 heifers

spread on the ground. On the most severe winter days, animals were unable to consume, only licked, the frozen, stone-hard silage.

In the second half of January, abortion showing a rapidly increasing tendency occurred in the purchased stock, reaching a peak of 8 to 10 abortions daily 2 weeks after the first case. The process mostly passed off in a month's time (Fig. 1). On the whole, 157 of the 200 purchased heifers aborted. Abortions occurred in the 5th to 9th, mostly in the 5th to 7th months, of pregnancy.

The abortions occurred on the pasture where the herd was kept. Since the symptoms of abortion were recognized late, isolation was not possible in due time. However, cows that had cast their fetuses, and the aborted fetuses and placentae were immediately removed from the herd.

Abortion did not seriously wear out the dams. About 30 to 40% of the cows retained the placenta for 1 to 3 days; in some cases the placenta had to be removed manually. The visible mucous membranes of the genital organs showed no appreciable changes.

With the majority of aborted animals, the involution of reproductive organs was rapid and uncomplicated. However, in a few cases treatment was necessary.

On the whole, 66 aborted fetuses and 3 placentae were sent to our laboratory.

Approximately half of the fetuses had inflammatory-necrotic foci in parenchymal organs, mainly in the liver and occasionally in the kidney. In some cases foci were observed macroscopically, but for the most part only by histopathological examination. Occasionally well-visible, in other cases only supposable, icterus was also observed. The examinations were largely hampered by the fact that the majority of fetuses arrived in frozen state and proved to be autolysed after thawing.

Part of the healthy calves delivered at full term succumbed to *E. coli* diarrhoea or coli septicaemia.

In herd B, the group kept close to the purchased (A) herd, and in the two herds kept on a more far-off farmstead (herds C and D), 5 to 10% of the pregnant heifers aborted in approximately the same period of pregnancy, coinciding in time with the abortions in herd A.

Microbiological and serological examinations

Bacteriological and histopathological examinations aimed at the detection of brucella, leptospira and chlamydia gave negative results with all fetuses. One fetus yielded Listeria, another streptococcus and a third staphylococcus, while 3 further fetuses yielded fungi.

IBR/IPV virus was isolated from 8 fetuses and herpesvirus virions were shown by electron microscopy in placentae examined in the Department of Pathology, University of Veterinary Science (Budapest) (Fig. 2).

Blood samples from 105 aborted cows were tested for leptospira and brucella antibodies. All of these, as well as the 10 blood samples tested for chlamydia antibodies proved negative. Five to 25 days after abortion, blood samples of 50 animals were tested for antibodies to IBR virus, and all showed considerable antibody titres (Table I). We did not test herd A serologically before the outbreak but as we have been informed the stock from which the heifers were transported was seronegative for antibodies to IBR/IPV.

Blood samples withdrawn from hearts of 22 fetuses proved to be negative for IBR/IPV antibodies.

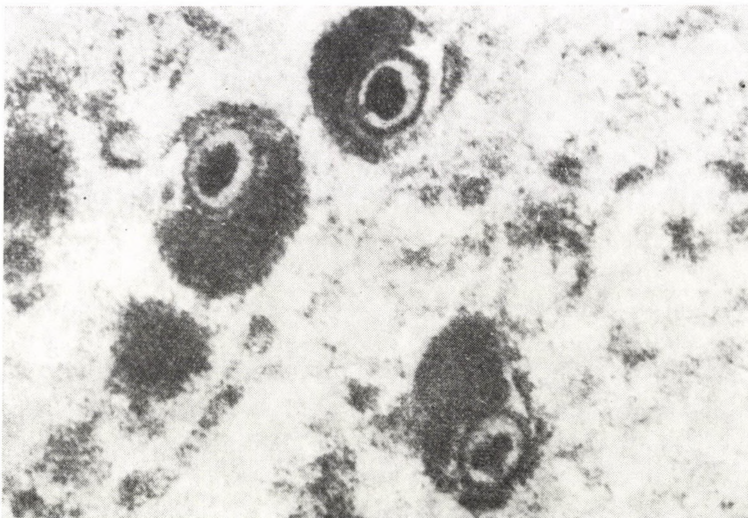

Fig. 2. IBR/IPV virus from a placenta. Electron micrograph, ×135,000

Table I

Virus-neutralizing (VN) titres to IBR/IPV virus in blood samples of 50 aborted animals (herd A) 5 to 25 days after abortion (against 100 $TCID_{50}$/0.1 ml virus)

Serum dilution	2	4	8	16	32	64	128	256
Distribution of positive samples by titre	3	4	3	9	11	12	7	1
Geometric mean titre					1 : 28			

Table II

Rate of seropositivity to IBR/IPV virus in herds C and D in relation to age and pregnancy

	Herd C	Herd D
Maiden breeding heifers	7/10*	5/10
Heifers in late pregnancy	8/10	9/10
Calved first-calf cows	10/10	10/10

* number of positive animals/number of animals tested

Virus-serological studies were extended to animals of herds C and D, i.e. maiden heifers and in-calf heifers in different stages of pregnancy. In these herds, though the number of seropositive animals rose with the advance of pregnancy, and with the passing-off of parturition (Table II), the rate of abortion was low. In these herds, attempts to isolate virus from the rarely occurring abortions failed.

Discussion

Of virus-induced abortions and reproductive disorders of cattle, those induced by herpesviruses, and particularly by the IBR/IPV virus, were identified first, and have been reported most frequently since (Draayer and Kirkbride, 1977; Straub, 1978, 1982).

Investigations into the outbreak described above have led us to conclude that the mass abortion must have been caused by the additive effect of the following factors. (i) High susceptibility of the transported heifers. At the time of their transportation to the farm, the first-calf heifers were, in all probability, seronegative. The "own" herds of the farm, being seropositive, appeared to be the reservoir of the virus. In these latter herds, only 5 to 10% of the animals aborted under conditions identical with those for the purchased stock. (ii) High exposure to infection. The animals were kept under loose conditions, in undi-

vided large groups, crowded in a small area. Their feed was spread on the ground for them, and neither calving stalls nor quarantine premises were available. (iii) Marked impairment of natural resistance. There is no doubt that in the severe, long winter, the feeding was unsatisfactory for the pregnant first-calf heifers which had no dry resting place and occasionally encountered difficulties with drinking water supply.

It is known from the literature that in herds with abortion even the calves delivered at full term are usually of low weight and are frequently affected by *E. coli* diarrhoea and coli septicaemia (Saxegard, 1970). This fact is confirmed also by our present observations.

Several authors have succeeded in inducing abortion in susceptible pregnant animals with virus strains isolated from IBR/IPV virus-induced abortions (Chow et al., 1964; Owen et al., 1964; Sattar et al., 1967; Straub, 1978, 1982).

It has been established that IBR/IPV virus can induce abortion in all periods of pregnancy. However, under natural conditions abortion occurs mostly in the second half of pregnancy, similarly to the present case.

Infection experiments have shown that no abortion occurs up to the 20th day post-infection (PI). Most abortions take place between the 20th and 35th day PI. In our case, filling-up of the stock and mixing of the two herds, i.e. infection, took place in the first half of December (Fig. 1).

According to the literature, multiplication of the virus in the organism leads to fetal death. However, the dead fetus is expulsed from the maternal organism only days after its death. Therefore, fetuses submitted for examination are often in a markedly autolysed state. Our observations agree with the above statement, although in our case the low ambient temperature would have certainly prevented autolysis taking place outside the dam's organism. Therefore, histopathological examination of fetuses was restricted only to detection, or in some cases only to premonition, of necrotic lesions. No intranuclear inclusion bodies were seen. This is in agreement with literary data suggesting that intranuclear inclusion bodies are infrequent, being present in large numbers only on the first days after virus infection, when they are easy to observe. Abortion, however, takes place only after this period.

The high serum antibody titres of aborted cows are consistent with most literary data (Straub, 1982).

Heart blood samples in a volume sufficient for testing were available from about one-third of the fetuses. All of these samples proved negative for antibodies to IBR/IPV virus. So far no seropositive fetal blood derived from natural cases of IBR/IPV has been reported in the literature. This can be explained by the fact that there is not enough time for antibody production even if the fetus is immunocompetent (Straub, 1982).

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OESOPHAGOGASTRIC ULCER IN SWINE AND VITAMIN U I. RELATIVE INCIDENCE OF THE SYNDROME IN HUNGARY

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Stomachs of 14,711 pigs of 95 to 105 kg live body mass and subjected to routine slaughter at slaughterhouses were examined to determine the relative incidence of oesophagogastric ulcer of swine in Hungary. The pigs originated from 48 pig farms.

The mucous membrane of the pars oesophagica of the stomachs was intact in only 6.8% of the pigs. While in 43.2% of the pigs only the epithelium showed lesions, in 50.0% of them erosions, ulcers or lesions indicative of ulcer healed with cicatrization were found.

The results were classified also on the basis of the pig farms where the animals had been kept, i.e. industrial large-scale units (I), units using semi-industrial technology (II) and units of conventional system (III). The mucous membrane of the pars oesophagica was intact in 3.5 (I), 6.8 (II) and 23.7 (III) % of the pigs. Pathological lesions of the epithelium were observed in 39.8 (I), 54.1 (II) and 54.4 (III) %, while erosions, ulcers or forms thereof healed with cicatrization in 56.7 (I), 39.1 (II) and 21.9 (III) % of the examined animals.

Keywords. Pig, oesophagogastric ulcer, relative incidence.

Of the two well-distinguishable forms of oesophagogastric ulcer of pigs, the long-known so-called peptic ulcer occurring in the glandular part (parr glandularis) of the stomach is negligible, due to its low incidence. Both literary data (Balogh et al., 1969; Milić et al., 1969; Muggenburg et al., 1964) and oui own experiences gained at slaughterhouses indicate that this form of gastric ulcer occurs at present rarely, in 0.4 to 2.1% of cases.

The situation is quite different with the so-called oesophagogastric ulcer of pigs, characterized by ulcer formation on the mucosa of the oesophageal part (pars oesophagica) of the stomach. In the last 15—20 years, parallel with the establishment of industrial-scale pig units and the development of swine industry, oesophagogastric ulcer has occurred with an ever increasing frequency, and on several occasions it has resulted in mass disease.

The opinion of investigators is almost fully unanimous in stating that oesophagogastric ulcer is absolutely distinct from the so-called peptic ulcer, i.e., these two forms of ulcer are unrelated. Only Nguyen Tan-Hung (1966) expressed the opinion that it was not necessary to discuss separately the ulcers localized in the pars oesophagica and those formed in the fundus of the stomach, since, in his opinion, peptic ulcer can be regarded as the acute, while oesophagogastric ulcer as the chronic, form of stomach ulcer of pigs.

Together with numerous authors, particularly with Dobos-Kovács et al. (1979), we consider oesophagogastric ulcer of pigs a new distinct syndrome, a

Fig. 1. Severe epithelial lesion (proliferation, para- or hyperkeratosis) extending to the whole pars oesophagica

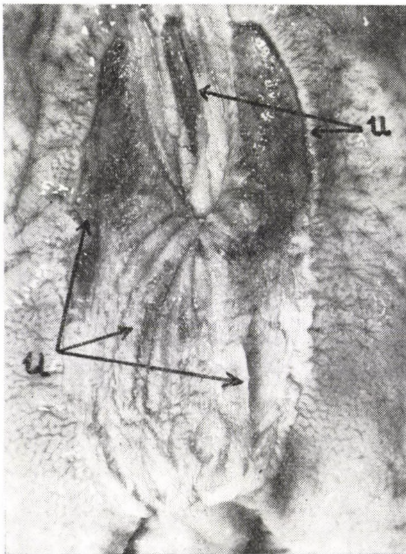

Fig. 2. Ulcers (U) of varying size and severity

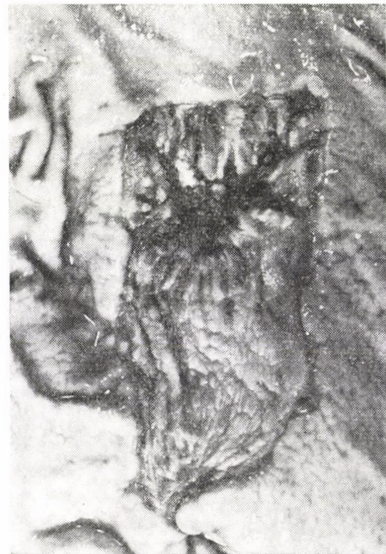

Fig. 3. Relatively superficial ulcer extending to the whole pars oesophagica

pathological process beginning with more or less severe epithelial lesions (proliferation, para- or hyperkeratosis) (Fig. 1) in the oesophageal part of the gastric mucosa. Subsequently, in addition to epithelial lesions of varying severity, erosions appear which develop into smaller or larger ulcers first restricted to the superficial, later extending also to the deeper tissues (Fig. 2). Sometimes these ulcers involve the entire oesophageal part of the stomach (Fig. 3). Due to these ulcers, recurrent bleeding and sudden death due to haemorrhage into the lumen of the stomach (Fig. 4) are not infrequent. Occasionally, perforation of the stomach wall may also occur. Ulcers may, however, heal by reparation, leaving behind a linear or starlet-shaped cicatrix. The contracting scar-tissue may deform the affected part of the stomach, frequently leading to constriction of the cardiac orifice (Fig. 5). The ulcerative process may recrudesce in the

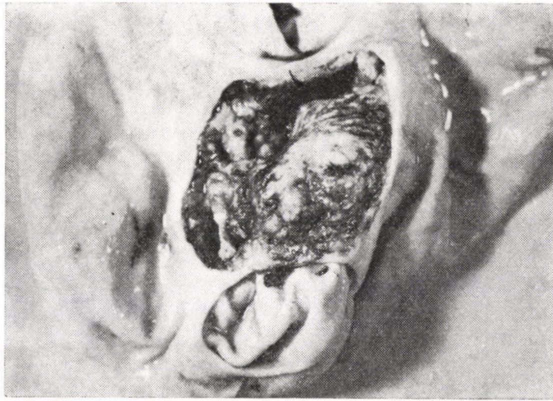

Fig. 4. Deep ulcer extending to the whole pars oesophagica. Fatal haemorrhage into the lumen of the stomach

Fig. 5. Ulcer healed with cicatrization. Constriction of the cardiac orifice (Oe)

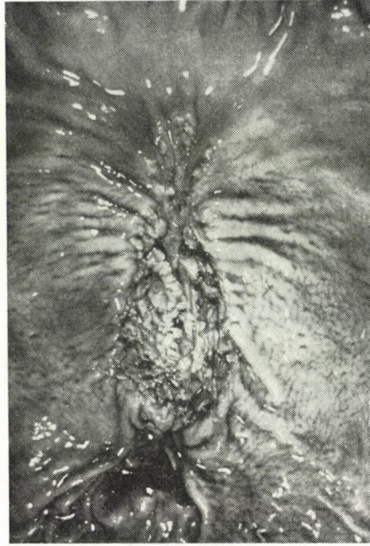

Fig. 6. Ulcer healed with cicatrization. The process has recrudesced in the newly-formed pavement epithelium

pavement epithelium formed in the area of the ulcer filled up by scar-tissue (Fig. 6).

Similar lesions may be found occasionally in the lower third of the oesophagus.

Almost all investigators attribute the first description of the disease to Bullard (1951). However, according to Asdrubali (1966), Mora observed oesophagogastric ulcer in Italy already in 1941.

In the last 10–15 years the syndrome has been found to occur, with varying frequency, in the pig population of almost all countries having an animal breeding more developed than average.

Its occurrence in Hungary was first reported in 1969 by Szabó and Süveges (1969) and Kovács (1969). The former authors remarked that oesophagogastric ulcer had been observed in Hungary already in 1963, however, it had not been described at that time.

Oesophagogastric ulcer may occur in pigs of all ages, in sucking pigs (Adamesteanu et al., 1969; Tamás et al., 1978) as well as in several years old breeding pigs (Kowalczyk et al., 1966; Szabó and Süveges, 1969). Nevertheless its highest incidence can be observed among fattened pigs. The numerous publications reporting on the average incidence of the syndrome also pointed out that oesophagogastric ulcer caused most damage in fattened pig populations.

According to reports published in the last 15–20 years, abroad the relative incidence of the syndrome was between 2.5 and 75.6% (Milič et al., 1969;

Muggenburg et al., 1964). However, in some experimental pig groups an incidence as high as 96.0% was observed (Sabec and Schröder, 1970).

Several publications have dealt with the relative incidence of oesophagogastric ulcer in Hungary as well. Szabó and Süveges (1969) examined 360 and 1000 fattened pigs at slaughter, and found ulcers and cicatrices in the pars oesophagica of the stomach in 11.4 and 10.2% of them, respectively. Kovács (1969) observed pronounced ulcerative lesions in the oesophageal part of the stomach in 10% of 2000 fattened pigs, while Balogh et al. (1969) found lesions in the pars oesophagica in 29.58% of the 1430 fattened pigs examined at slaughter. In the latter group of pigs, the observed lesions showed the following distribution; pathological lesions restricted to the epithelium were found in 28.88, erosions in 0.63, and ulcers in 0.07% of the cases. The authors pointed out that the average incidence showed considerably wide variation (between 15 and 49%) between pig herds.

Kovács (1974) reported that of 13,432 fattened pigs examined at 8 slaughterhouses of Hungary in the period between 1967 and 1972, 1712 animals (12.74%) had pronounced ulcers in the pars oesophagica of the stomach. The relative incidence showed rather wide variation (8.6 to 20.2%) between slaughterhouses. On the basis of his investigations, Kovács accepted the prognostic supposition that the ulcerative form of the disease might reach an average incidence of 20% by 1975–1976, and 25% by 1980, in industrial, large-scale fattened pig units of Hungary.

Since part of the above authors (Kovács, 1969, 1974; Szabó and Süveges, 1969) have studied only cases of pronounced ulcer formation, and others (Balogh et al., 1969) the relative incidence of the more or less progressed stages, i.e. lesions characteristic of the syndrome and well-distinguishable by gross pathological examination, we (Tamás et al., 1977; Tamás and Bokori, 1979), agreeing with the latter authors, attempted to assess the relative incidence of oesophagogastric ulcer in Hungary.

In the present paper, results obtained by examining stomachs of 14,711 pigs are reported.

Materials and methods

On the whole, stomachs of 14,711 pigs of 95 to 105 kg live body mass, originating from 48 pig farms and slaughtered as usual at slaughterhouses were examined to determine precisely the relative incidence of oesophagogastric ulcer in Hungary on the basis of gross pathological findings obtained by examining large numbers of animals.

Lesions found in the pars oesophagica of stomachs examined by dissection were classified into six groups as follows;

Group 1. The mucosa was free from pathological changes.

Group 2. There were only mild, circumscribed epithelial lesions restricted to the edges of mucosal folds.

Group 3. The mucosa exhibited severe, diffuse lesions of the epithelium.

Group 4. In addition to epithelial lesions of varying severity, erosions the size of a pinhead or of a barley-corn were also present.

Group 5. One or more acute, subacute or chronic ulcers the size of a cornel grain or of a child's palm were observed.

Group 6. Marks of ulcers healed with cicatrization were seen.

The pigs examined had come from industrial large-scale pig farms of closed system, from semi-industrial units of incompletely closed system, and from those operating with a conventional management technology.

Results and discussion

The results of the postmortem examination of stomachs of 14,711 pigs are summarized in Table I.

Data obtained during the present survey were classified also on the basis whether the examined pigs had come from farms of industrial, semi-industrial or conventional system. The results in this classification are shown in Table II.

Table I shows that it is not without reason that oesophagogastric ulcer of pigs has come to the limelight in Hungary, since of the examined 14,711 pig stomachs only 1003 (6.8%) had intact mucous membrane in the pars oesophagica. The oesophageal part of the stomach showed smaller or larger erosions, acute, subacute or chronic ulcers of varying severity, or mucosal lesions healed with cicatrization in 7356 animals (50.0%).

The gross pathological findings of those 6352 pig stomachs (43.2%) which showed "only" epithelial lesions of greater or lesser severity are also remarkable and must be considered serious in respect of the pathological course. Namely,

Table I

Results of stomach examinations performed at slaughterhouses

Lesions found in the pars oesophagica of the stomach	Number of animals	%
Mucosa free of pathological changes	1003	6.8
Circumscribed, mild lesions in the epithelium	2611	17.8
Severe, diffuse lesions in the epithelium	3741	25.4
Epithelial lesions with erosion(s)	2678	18.2
Ulcer(s)	3619	24.6
Ulcer healed with cicatrization	1059	7.2
	14711	100.0

Table II

Results of stomach examinations in pigs derived from units of different management technology

Lesions found in the pars oesophagica of the stomach	Industrial-scale units		Semi-industrial units		Conventional units	
	number of animals	%	number of animals	%	number of animals	%
Mucosa free of pathological changes	392	3.5	76	6.8	535	23.7
Circumscribed, mild lesions in the epithelium	1791	15.8	230	20.5	590	26.1
Severe, diffuse lesions in the epithelium	2724	24.0	377	33.6	640	28.3
Epithelial lesions with erosion(s)	2265	20.0	183	16.3	230	10.2
Ulcer(s)	3219	28.4	193	17.2	207	9.2
Ulcer healed with cicatrization	940	8.3	63	5.6	56	2.5
	11331	100.0	1122	100.0	2258	100.0

according to the studies of Adamesteanu et al., 1969; Asdrubali, 1966; Dobos-Kovács et al., 1979; Kowalczyk et al., 1960; Muggenburg et al., 1964; Perry et al., 1963; Rothenbacher et al., 1963; Szabó and Süveges, 1969, and Tamás et al., 1977 and 1978, these primary lesions can be regarded as the initial stage of the syndrome. It is merely a question of time, but even more so a matter of the presence of unfavourable environmental factors, in most cases faults of management technology, that the syndrome develops to its most severe stage, i.e. death by haemorrhage into the lumen of the stomach. The unfavourable effects of the environment (stress factors) may largely contribute to the aggravation of the disease which has been induced primarily by another factor, or factors (Barzoi et al., 1968; Curtin et al., 1963; Dobos-Kovács et al., 1979; Kovács, 1976; Reese et al., 1966; Riker et al., 1967; Tamás and Bokori, 1979; and Tamás et al., 1978). However, it should be emphasized that, according to our observations, stress is only an aggravating, and not a primary causative, factor in oesophagogastric ulcer of swine.

The data of Table II also indicate that oesophagogastric ulcer of pigs occurs significantly more frequently in fattened pig populations kept in industrial-scale farms than in those of conventional ones. While in conventional farms the average incidence was 76.3% (extreme values 36.0 and 97.5%), in industrial-scale units this value was 96.5% (extreme values 89.6 and 100.0%). The average incidence found in slaughtered pigs originating from semi-industrial fattening farms (93.2%) was between the above two values.

In addition to the relative incidence of the syndrome, the more severe form characterized by ulcer formation was also more frequent in industrial

pig units (28.4%) than in conventional farms (9.2%). In semi-industrial farms the frequency of stomach lesions showed an intermediate value (17.2%).

Therefore, we can state that although oesophagogastric ulcer of pigs occurs in practically all pig farms of Hungary, it causes the most severe economic losses in large-scale units operating in an industrial system. On the basis of our investigations the prognosis proposed by Kovács (1974) seems not to be exaggerated. Namely, he expected that the ulcerative form of the syndrome might reach an average incidence of 20% by 1975–1976, and 25% by 1980, in large-scale pig stocks operating in an industrial system. The present authors (Tamás et al., 1977) found the incidence of such lesions at 23.7% in a survey conducted in 1976, and 28.4% during the present survey.

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OESOPHAGOGASTRIC ULCER IN SWINE AND VITAMIN U

II. NATURE OF VITAMIN U

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The discovery, isolation, distribution in nature, microbiological activity, biosynthesis, physicochemical properties, toxico-pharmacology and analytical methods for determination, of vitamin U are outlined.

The use of vitamin U in the treatment of ulcerative diseases and its role in the treatment of the oesophagogastric ulcer syndrome of swine are discussed.

Keywords. Vitamin U, treatment, oesophagogastric ulcer, pig.

Salts of methylmethionine-sulfonium were synthesized long ago (Toennies and Kolb, 1945), but they did not attract attention until they were discovered in plants and until it was shown that the therapeutical anti-ulcer effect of vegetable juices was largely conditioned by the presence of these compounds within them.

Cheney (1940), who was the first to discover the anti-ulcer effect of certain food products (vegetable juices), supposed that gastroduodenal ulcer was caused by deficiency of a definitive nutritional factor, which he attributed to the category of vitamins. Therefore, salts of methylmethionine sulfonium have been called vitamin U (from the Latin ulcus) as proposed by Cheney.

McRorie et al. (1954*a*) established that the spread and thermolability of the methylsulfonium derivative of methionine coincided with those of the anti-ulcer factor of vegetables which had been reported by Cheney. This gave the final impetus to testing synthesized salts of methylmethionine-sulfonium for anti-ulcer properties. Both experimental and clinical studies have been conducted. The crystalline powder methylmethionine-sulfoniumchloride was found to be more than thousand times as active as the initial cabbage juice itself.

Occurrence of vitamin U

Vitamin U is a widely-spread component of plant-tissues the biosynthesis of which is mediated by S-adenosyl-methionine. The presence of vitamin U has been established in extracts of a number of plants including green parsley, cabbage, turnip, pepper, carrots, onions, lettuce, asparagus, tomatoes, etc.

Wong and Carson (1966) have, for instance, isolated S-methyl-methionine-sulfonium salt (SMM) from fresh tomatoes. The identity of this salt was proved by infrared spectra, melting points and paper chromatograms. The compound decomposed to yield homoserine and dimethyl sulfide. The behaviour of the isolated sulfonium salt and its concentration in the tomato suggest that it is an important precursor of tomato aroma as well. This has been confirmed by Miers (1966).

Challenger and Hayward (1954) demonstrated a methylsulfonium derivative of methionine in asparagus.

In Hungary, Berndorferné-Kraszner et al. (1976) demonstrated the occurrence and distribution of vitamin U in some corn hybrids. They emphasized the role of vitamin U in aroma formation of corn. Telegdy-Kováts et al. (1974) stressed the richness of Hungarian cultural plants in vitamin U.

Microbiological activity of methylmethionine-sulfonium salts

The dimethylsulfonium derivative of methionine can replace methionine for a variety of microbes. This compound was found to be 3 times as active as methionine in reversing the toxicity of sulfanilamide and in promoting the growth of certain strains of *Escherichia coli*, *Lactobacillus arabinosus* and *L. casei* (McRorie et al., 1954*b*). *Streptococcus faecalis* R and *Leuconostoc mesenteroides* P-60 do not utilize the sulfonium derivative of methionine. The enhanced activity of the methylsulfonium derivative of methionine is more pronounced under conditions of restricted single-carbon-unit metabolism.

Growth responses to various methionine analogues of *Lactobacillus plantarum* ATCC 8014 are demonstrated in Fig. 1 (Hegedűs et al., 1977*a*). The effect

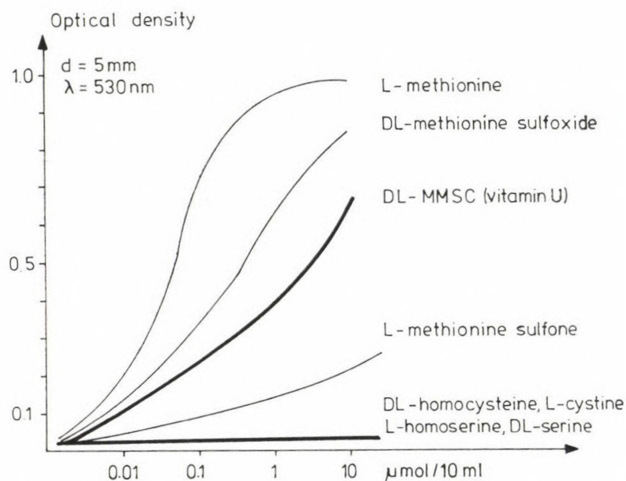

Fig. 1. Growth responses to various methionine analogues of *Lactobacillus plantarum* ATCC 8014

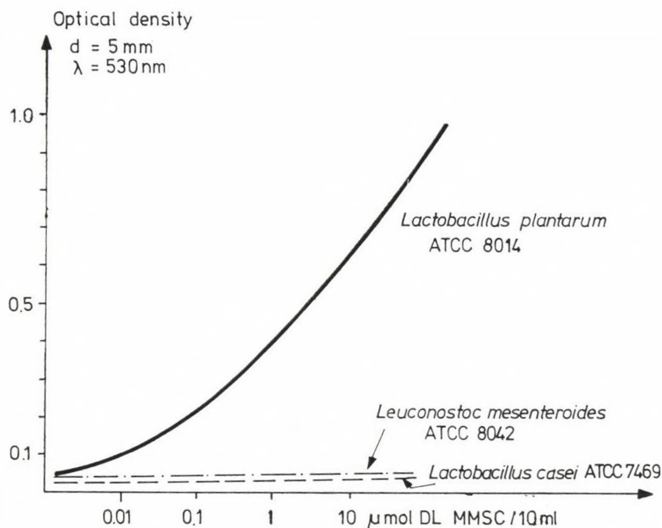

Fig. 2. **Growth responses to MMSC (vitamin U) of different bacteria in Bacto Methionine assay medium (Difco)**

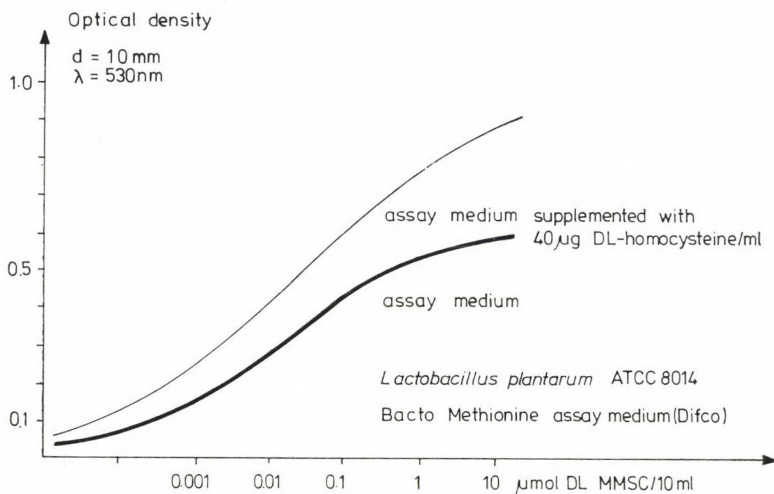

Fig. 3. **Effect of addition of homocysteine to MMSC (vitamin U)**

of S-methyl-methionine in replacing methionine in nutrient media of different test strains as shown by us is displayed in Fig. 2. The sensitivity of growth response of *Lactobacillus plantarum* can be enhanced by addition of homocysteine to the assay medium (Fig. 3). This can be utilized for microbiological assay of vitamin U (Hegedűs, 1977).

Biosynthesis of vitamin U and its biochemical role

The biosynthesis of vitamin U takes place from methionine with the mediation of S-adenosyl-methionine (Fig. 4) (Shapiro, 1956).

S-methyl-methionine is a very active natural methyl donor because of its rapid utilization. A combination of homocysteine and S-methyl-methionine replaces methionine for growth in many bacteria, thus vitamin U takes place in the biosynthesis of methionine from homocysteine (Durell and Sturtevant, 1957; Shapiro, 1955). This is demonstrated by the wide distribution of S-methyl-methionine and S-adenosyl-methionine-homocysteine transmethylase in plants (Shapiro and Yphantis, 1959; Turner and Shapiro, 1961). The metabolic pathways of methionine to illustrate these reactions are shown in Fig. 5.

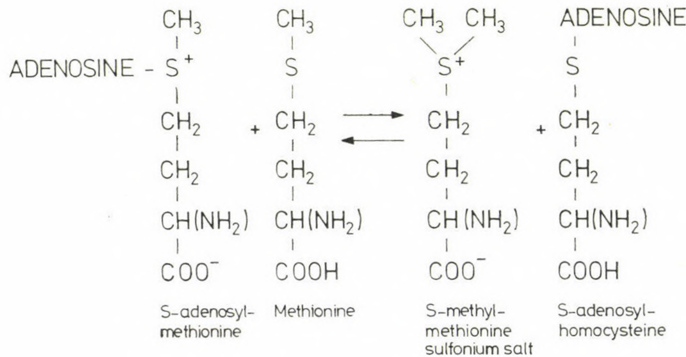

Fig. 4. Biosynthesis of vitamin U

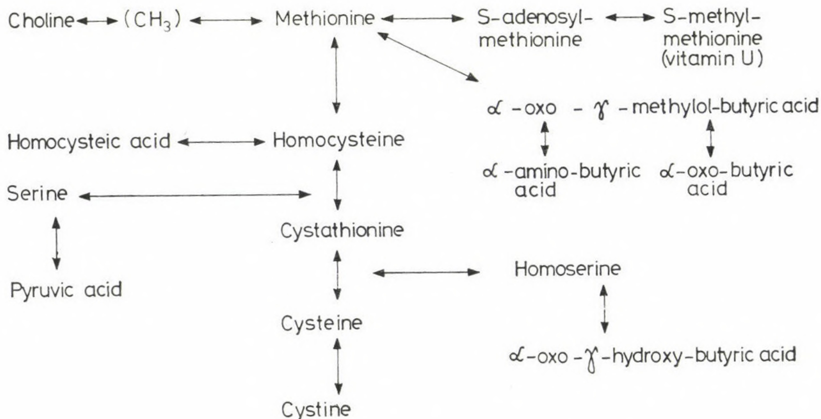

Fig. 5. Metabolic pathways of methionine

Physicochemical properties and toxico-pharmacology

The melting point of the crystalline powder of DL-methionine-methyl-sulfonium-chloride (MMSC) is 139–140 °C; 1 g MMSC in 10 ml water gives a clear colourless solution. In a dry state it is relatively stable, but during long storage of up to one year or longer it may undergo decomposition with the appearance of yellow colour and the characteristic odour of dimethyl sulfide. High temperature accelerates this process. The testing of the stability of S-methyl-methionine sulfonium-chloride in the form of a 10% solution indicated destruction after 6 h at 37 °C; the rate of decomposition was 2% in water, 8% in 0.02 N HCl and 11% in gastric juice (Bersin et al., 1956).

Table I

Toxicity of methylmethionine-sulfonium salts for mice

Anion	1 day LD_{50} per os (g/kg body weight)	10 days LD_{50} per os (g/kg body weight)
Chloride	8.9	8.4
Sulphate	6.7	6.7

Methylsulfonium salts are easily formed from methionine by phosphotungstic acid (Floyd and Lavine, 1954). Methyl, ethyl, propyl and isopropyl alcohols in 50% (w/w) sulfuric acid react with methionine, and the corresponding sulfonium salts are readily formed (Lavine et al., 1954).

Decomposition of the methionine-methylsulfonium ion in acid solutions yields methionine; the rate and extent of the reaction depends on the nature of the acid present. In hot neutral or alkaline solution, dimethyl sulfide is formed. Solutions of methionine-methylsulfonium salts can be regarded as relatively stable at ordinary temperatures and even alkaline solutions can be distilled in vacuum without decomposition.

Salts of methylmethionine-sulfonium are well tolerated by animals and their lethal doses are very high. According to data by Bersin et al. (1956) the toxicity of methylmethionine-sulfonium salts for mice was as indicated in Table I.

With the highest doses, experimental animals soon develop transient symptoms, such as disturbances in coordination, bronchial spasm and a weakening of respiration.

The acute toxicity of methylmethionine-sulfonium-chloride was determined on albino mice weighing 17–22 g on i.v. injections of the preparation. The DMT (dose of maximal tolerance) of the preparation was 2 g/kg body weight. $LD_{50} = 2.76 \pm 0.04$ g/kg; $LD_{100} = 4$ g/kg (Bukin and Anisimov, 1973). These results ascribe vitamin U to a slightly toxic compound.

The picture of poisoning of the experimental animals was expressed in high respiration rate and ataxia. The animals died in 1–5 min with clonicotonic convulsions.

Rabbits and rats were given multiple injections of the preparation. In experimental and control rabbits leucocytes, erythrocytes and the leucocyte count were investigated. The results showed no evidence that the morphological indices have changed. Pathomorphological examination at autopsy revealed no deviations from the normal picture.

Analytical methods for vitamin U

Microbiological determination of vitamin U is based on the measurement of methionine activity. Test organisms suitable for determination of methionine were therefore examined for ability to utilize MMSC for methionine synthesis in methionine-deficient medium. *Lactobacillus plantarum* ATCC 8014 gave growth response to MMSC, and the response could be considerably enhanced by addition of DL-homocysteine to the medium. *L. plantarum* ATCC 8014 in the presence of MMSC transforms homocysteine by methylizing it to methionine. This methionine activity is proportional with the quantity of S-methylmethionine sulfonium-chloride and can be measured by adding it to methionine-free assay medium (Hegedűs et al., 1977).

Determination of vitamin U concentrations by automatic amino acid analyser was described by Skodak et al. (1965).

Separation of MMSC by a two-dimensional thin-layer ion exchange chromatography on "Fixion 50×8" precoated cation exchanger plates (Chinoin, Budapest) was published by Hegedűs et al. (1977).

The use of vitamin U in the treatment of ulcerative diseases

Szabó and Vargha (1960) investigated the effect of vitamin U (Vit-U-Pept-Magen-Darm-Tabletten) on gastric ulcers caused by application of phenylquinoline carboxylic acid (PCC) in dogs. The following effects were observed: the formation of erosions and gastric ulcers induced by PCC was inhibited by vitamin U. Vitamin U induced changes in the glandular epithelial cell layer: parietal cells were found smaller and reduced in number, whereas the number of chief cells was found increased. This process may be of importance in causing chemical alterations in gastric secretion. The production of mucus which is generally observed in the vicinity of ulcer was reduced in the stomach of animals treated with vitamin U, as compared with controls. Experiments performed with injection of Chinese ink showed deposition of ink in ulcer and erosions to be considerably less if PCC and vitamin U had been applied simultaneously than after sole application of PCC.

Bersin et al. (1956), who studied the therapeutic effect of methylmethionine-sulfonium salts, found that it influenced the intestinal and liver function favourably. The application of MMSC in the treatment of ulcerative disease was also favourable; the daily dose of the preparation was 250–300 mg, the treatment lasted 35–90 days.

Observations are available on the use of vitamin U in patients with ulcerative disease under policlinical conditions. Nakamura (1965) gave daily 150–450 mg vitamin U to 18 patients with gastric ulcer. Subjective symptoms of the disease passed in 91% of cases.

The symptom of the "niche" disappeared after an average of 33 days in 87% of cases. These results were highly promising for the treatment of ulcerative disease in combination with acid-decreasing and other remedies.

The absence of untoward effects after the use of vitamin U was remarkable. Except for complaints of an unusual odour (smell of cabbage) no untoward effects were noted. Some authors, however, emphasize that the treatment of ulcerative disease by the end of the 3rd week noted complete cessation of the epigastric pains as well as cessation of heartburn, belching, nausea and coating of the tongue. These symptoms usually disappeared within the first 5 days. In patients with ulcerative disease running its course in combination with chronic hepato-cholecystitis, gastroduodenitis, colitis and those with a history of gastric surgery, the above-mentioned symptoms ceased later.

The clinical investigations have shown that a treatment with vitamin U tablets, 250–300 mg vitamin/day for 28–40 days resulted in a considerable amelioration in the diseased condition viz. the pain and the dyspeptic syndromes ceased; the motor, acid-secreting and secretory functions of the stomach and intestine normalized; the enzymatic functions of the liver improved; the "niche" symptom disappeared; antihistamine and antiserotonine effects appeared and the protein spectrum of the blood improved.

Concurrent diseases, however, reduced the therapeutic effect of vitamin U. Side effects (nausea, vomiting, enhanced pains) were extremely rarely observed. In such cases, reduction of the dose eliminated the complaints.

The role of vitamin U in the treatment of oesophagogastric ulcer syndrome in swine

Peptic ulcers in the stomach of swine restricted to the glandular part are rare. Oesophageal ulcer in the oesophageal part of the swine stomach occurs more frequently. The frequency and economic importance of this syndrome in Hungary has been outlined by Tamás et al. (1983) (see the accompanying paper, p. 145).

Despite the fact that a large number of publications deal with the aetiology of the syndrome, the exact causes of oesophagogastric ulcer are not yet known. Some of the authors suggest that the oesophagogastric ulcer syndrome

of swine may be caused by several factors jointly or separately. The causes should be looked for in fattening and feeding technology, in the physical state, form and composition of the feed mixture, and in external environmental factors.

It is probable that preventive or therapeutic attempts considering only one of these factors do not lead to unequivocal results; these attempts often lead to the contrary of the desired results. The best way would be the use of an additive in the feed mixtures to prevent or reduce the development of oesophagogastric ulcer in swine (Hegedűs et al., 1977).

Vitamin U has been thought to be an effective additive. We investigated the possibility of preventing or hindering the development of oesophagogastric ulcer by vitamin U supplementation of concentrates. The production technology of premixes supplemented with vitamin U has been patented (Hegedűs et al., 1979).

According to our experiences, supplementation of pig feeds with vitamin U can be summarized as follows: (i) it reduces the overall losses, (ii) prevents more severe oesophagogastric ulcers from being developed, (iii) accelerates the reparation of ulcers, and (iv) increases the economic yield of fattening pigs.

Detailed information will be published in future papers.

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HAEMODYNAMIC RESPONSES TO EXPERIMENTAL SEPTIC SHOCK IN CALVES

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Induction of septic shock in 20 calves by strangulating a segment of jejunum elicited various haemodynamic responses. Hypodynamic response dominated (60%) over hyperdynamic (15%) and mixed responses (25%). Cardiac output and cardiac index decreased at early and late/terminal shock stages in hypodynamic, while the reverse was observed in hyperdynamic. In the mixed group, there was increase in these parameters at early and decrease at late shock stages. Progressive rise in heart rate and decline in mean arterial pressure and central venous pressure correlated with the different haemodynamics. Stroke volume and total peripheral resistance decreased at both the early and the late shock stages, though the magnitude of their decline varied considerably with different haemodynamic responses.

Keywords. Haemodynamic response, experimental septic shock, calf.

Septic shock is encountered frequently in veterinary practice in a number of conditions in which, due to septicaemia, endotoxin is released. In spite of the information available for small animals and human beings, this syndrome remains poorly-defined in bovine species, where very few studies have been conducted so far (Singh and Kohli, 1980). Most of the experimental animal models of septic shock have involved injection of bacterial endotoxin or live bacterium directly into the blood stream (Gahhos et al., 1981), but the picture of shock produced in this way is markedly variant from clinical cases of septic shock (Perbellini et al., 1978).

In the management of shock, alleviation of disturbed circulatory state is one of the major aims. Tissue perfusion highly depends on cardiac output, its distribution and other haemodynamic parameters (Kho and Shoemaker, 1968). The haemodynamic aspects of shock have been investigated in other species, but not in bovines. Extrapolation of data from the other species would be misleading as considerable species variation in manifestation of shock has been reported (Tikoff et al., 1966; Singh and Kohli, 1980). The present paper reports the variations observed in the haemodynamic responses at different stages of experimentally induced septic shock in calves.

Materials and methods

This study was conducted on 20 clinically healthy crossbred calves of 1 to 1.5 years age. Before the experiments the animals were fasted overnight. After securing the animals comfortably in left lateral recumbency, siliastic catheters were placed, under local anaesthesia, in the carotid artery and the jugular vein for recording mean arterial pressure (MAP) and central venous pressure (CVP), respectively. A three-way stop-cock was attached to the catheters for facilitating the injection of dye and collection of blood samples for cardiac output (Q) measurements. Septic shock was induced by strangulating a 50–55 cm segment of jejunum by the technique published from this laboratory (Singh and Kohli, 1980).

Cardiac output was measured using Evans blue dye (T–1824) by the technique of Stowe and Good (1960), except that the arterial blood samples were collected from the carotid artery. It was calculated according to the formula:

$$Q = \frac{60 \times A}{T \times C \times (1 - H)},$$

where Q = cardiac output in litre/min; A = the amount of dye injected, in mg; T = the time in seconds between the first appearance of the dye and its reappearance following recirculation; C = the average concentration of dye in mg/l of plasma, and H = the haematocrit value.

Q was also calculated for kg body weight, i.e. ml/min/kg. Cardiac index (Q/body surface, m^2) was calculated as the product of Q l/min and body surface area. The latter was measured by Meeh's formula;

$$A = K \times W^{2/3},$$

where A = body surface area; K = a constant factor, 0.015 for calves; and W = weight of the animal.

Stroke volume was measured as a product of Q/ml/min and heart rate/min. Total peripheral resistance (TPR) was calculated according to the formula:

$$TPR = \frac{\text{MAP mmHg}}{\text{Q/ml/kg/min}}$$

and expressed in peripheral resistance units (PRU).

The MAP was measured in a mercury manometer while CVP was recorded against a vertical saline column. Heart rate (HR) was recorded from a cardiac telemeter with base apex leads. These were monitored at short intervals till termination of the experiments.

The stabilized preshock value of each parameter served as normal control (stage 0). Since survival time of individual animals following shock varied widely, the time elapsing between induction of shock and death was divided into five stages of equal duration (stages 1 to 5). In the following, the designation early shock stage indicates stage 2 and terminal shock stage indicates stage 5. Statistical analysis of data was done with one-way analysis of variance, and for stagewise analysis Student's *t* test was employed.

Results

There were variations in the haemodynamic responses after induction of septic shock. Twelve animals manifested hypokinetic haemodynamics in which cardiac output and the related parameters decreased throughout the period of study. Contrary to this, three animals exhibited a hyperkinetic feature with an increase in the cardiac output. In the remaining five animals cardiac output exhibited mixed dynamics, i.e. it was hyperkinetic at early shock stages followed by hypokinetic dynamics at late stages. The average survival time after induction of shock in the hypokinetic, hyperkinetic and mixed group was 22, 27 and 29 h, respectively. Mean values $\pm$ SE of different parameters are presented in Table I.

(A) Hypodynamic response: Average cardiac output (ml/kg/min) and cardiac index decreased significantly ($P < 0.05$) and highly significantly ($P < 0.01$) at the early and the terminal stages, respectively. Stroke volume exhibited a fall at both the stages (Table I). TPR showed a slight tendency of fall in the early shock stage, except for three animals, which exhibited an increase at the early stage. Contrarily, in the late shock stage the fall in TPR was statistically significant and uniform in all the animals. The HR showed a consistent and progressive increase, whereas MAP exhibited a gradual fall. The mean values of CVP declined gradually. All these overall variations were highly significant ($P < 0.01$).

(B) Hyperdynamic response: Average cardiac output increased from 9.47 l/min to 10.62 l/min and 11.64 l/min in the early and terminal shock stage, respectively. Cardiac index manifested similar rises at both stages. Stroke volume exhibited a fall but of lesser magnitude than in hypokinetic shock. The fall in the TPR was appreciable at both stages and the manifestations of HR, MAP and CVP differed widely in magnitude (Table I) compared with the hypokinetic group.

(C) Combination of hypo- and hyperdynamic response: Cardiac output exhibited a mixed pattern of kinetic function. In these animals Q was hyperkinetic at the early shock stages and hypokinetic at the late/terminal stages. Stroke volume declined uniformly, except in one animal, in which following

Table I

Mean ±SE of haemodynamic parameters in hypokinetic (A), hyperkinetic (B) and combination of hypo- and hyperkinetic (C) septic shock in calves

Parameter	Stages of shock					
	0	1	2	3	4	5
MAP, Hgmm						
A	122.4	116.0	100.0	98.0	85.8	68.0
	±3.1	±3.6	±2.7	±2.9	±3.7	±3.9
B	120.0	111.6	105.3	99.6	87.6	80.0
	±6.8	±6.3	±7.5	±8.2	±3.9	±8.9
C	116.4	106.2	99.0	95.0	86.6	60.0
	±4.1	±5.3	±5.0	±6.5	±5.0	±6.1
CVP, cm H_2O						
A	6.3	5.3	4.2	1.0	—0.70	—2.3
	±0.72	±0.98	±1.06	±0.52	±0.29	±0.24
B	6.1	5.6	3.0	1.06	0.93	0.76
	±1.26	±1.70	±0.86	±0.06	±0.7	±1.13
C	4.5	3.1	0.90	—0.30	—0.06	—1.80
	±0.43	±0.56	±0.77	±0.97	±1.04	±1.40
Heart rate						
A	62	72	80	95	103	128
	±2.1	±4.9	±4.6	±6.7	±7.7	±11.0
B	65	69	78	89	94	120
	±2.4	±4.8	±6.1	±2.1	±6.3	±15.3
C	68	82	103	118	118	138
	±5.9	±5.1	±4.3	±5.8	±8.8	±4.0
Cardiac output, l/min						
A	10.60	9.85	9.20	8.61	8.32	8.01
	±0.45	±0.51	±0.35	±0.34	±0.35	±0.33
B	9.47	10.04	10.62	11.13	11.38	11.64
	±0.97	±1.21	±1.47	±1.29	±1.37	±1.48
C	11.6	11.71	12.70	11.58	11.01	10.44
	±1.66	±1.10	±1.10	±0.96	±1.57	±2.20
Cardiac output, ml/kg/min						
A	115.30	108.1	99.6	93.7	90.8	87.7
	±2.9	±3.4	±4.0	±3.6	±3.7	±3.4
B	111.4	118.7	124.2	130.5	134.2	137.0
	±7.5	±7.9	±9.6	±11.2	±12.3	±14.1
C	115.0	117.0	125.8	115.6	108.8	102.0
	±13.7	±10.2	±11.3	± 4.9	±10.6	±19.2

Table I continued

Parameter	Stages of shock					
	0	1	2	3	4	5
Cardiac index, l/min/m²						
A	5.00	4.64	4.33	40.6	3.92	3.77
	±0.13	±0.15	±0.14	±0.16	±0.12	±0.11
B	4.79	5.08	5.37	5.62	5.75	5.89
	±0.28	±0.30	±0.31	±0.29	±0.31	±0.4
C	5.00	5.04	5.47	4.99	4.74	4.5
	±0.63	±0.45	±0.45	±0.31	±0.50	±0.71
Stroke volume, ml/beat						
A	170.9	136.8	115.0	90.6	80.8	62.6
	±8.2	±7.6	±8.1	±9.1	±7.7	±6.5
B	145.6	145.5	136.1	125.0	121.1	97.0
	±18.2	±19.1	±16.3	±12.4	±10.6	±10.3
C	170.5	142.8	123.3	98.1	93.3	75.6
	±25.2	±21.4	±17.6	±9.9	±16.3	±13.2
Peripheral resistance						
A	1.06	1.06	1.00	1.04	0.94	0.77
	±0.04	±0.03	±0.05	±0.06	±0.03	±0.03
B	1.07	0.94	0.85	0.76	0.65	0.58
	±0.12	±0.13	±0.12	±0.12	±0.14	±0.12
C	1.01	0.91	0.79	0.82	0.79	0.62
	±0.13	±0.09	±0.08	±0.08	±0.08	±0.06

substantial decrease at early shock, it approximated the preshock level. There was a non-significant fall in TPR. The overall variations in the HR, MAP and CVP were statistically highly significant ($P < 0.01$) and the magnitude differed from the previous two groups (Table I).

Discussion

It has been convincingly demonstrated that septic shock can be induced experimentally by strangulating a segment of bowel (Singh and Kohli, 1980). The septic shock thus induced in the present study elicited hypokinetic or hyperkinetic haemodynamics. In man, the cardiac index in early septic shock was found to be increased or normal in the majority of the cases (Lillehei, 1971), and in dogs hyperkinetic shock predominates (Perbellini et al., 1978). In contrast, in the calves of the present study hypokinetic septic shock was present in 60% while hyperkinetic in only 15%. In the remaining 25% a

combination of both was observed. These differences in the manifestation of septic shock suggest striking species variation. The average survival time after induction of shock suggests that the hypodynamic state of septic shock was more detrimental to calves than the hyperdynamic response. This is contrary to the findings in man, where a hyperkinetic circulation associated with hypotension was found to be more detrimental (Cohn et al., 1968). However, a longer survival time has also been observed in dogs with hyperkinetic septic shock (Perbellini et al., 1978). The longest survival time in the calves with combination of low and high flow states is poorly explained on the basis of the results obtained. It is possible that the beneficial effects rendered by high flow state, due to hyperdynamic response, took a comparatively longer time to be offset by the subsequent hypodynamic circulation.

The progressive rise in the HR at the terminal stage in hyperkinetic shock (87.5%) was considerably less when compared to the hypokinetic (106.5%) and mixed shock (102.9%). This variation in tachycardia was probably the first determinant which affected the haemodynamic responses in the three forms of shock. The varying intensity of tachycardia in the three forms of shock could be well related to the degree of hypotension which was maximum (48.5%) in the mixed group, followed by the hypokinetic (44.4%) and the hyperkinetic (33.3%) groups at the terminal stage. The fall in CVP in the mixed (140%), hypokinetic (136.5%) and hyperkinetic (87.5%) groups also correlated with the degree of hypotension and the changes in cardiac output. The relationship of Q with varying amounts of venous return, reflected by CVP, has been established (Guyton, 1955). The CVP is the most convenient and most reliable index of the circulating blood volume (Hamit, 1965) and it gives a better judgement of the prevailing status of the shock as well as a clear picture of haemodynamic alterations (Wilson et al., 1971). On the basis of this study, it is proposed that a greater decline in CVP in face of significant hypotension should markedly reduce the venous return and thus affect the working capacity of the heart. A decrease in the cardiac output takes place if the HR is increased significantly in the presence of decreasing atrial pressure (Sugimoto et al., 1966). This results in an impaired ventricular filling and a diminished systolic ejection. Further, there is a linear relationship between pressure and flow rates in the systemic circulation (Read et al., 1957). Therefore, in the present study the markedly increased HR, along with greater hypotension and significant decline in CVP, was the chief cause of decrease in cardiac output in hypodynamic septic shock. Since cardiac index is an expression of Q in relation to body surface area, the former declined in tune with Q l/min. Stroke volume, on the contrary, is inversely related to HR for a given volume of blood ejected in a unit of time. The greater increase in HR coupled with fall in Q was the cause of higher decrease (58%) in stroke volume in the late shock stages than in the early stages (41%).

Increased cardiac output in the early stages of hyperkinetic septic shock in man has been attributed to direct effects of bacterial endotoxin, hyperthermia and increased body metabolism, while in the middle and late stages it is due to pulmonary insufficiency, pulmonary venous admixture, acidosis, etc. (Shoemaker et al., 1970). Apart from the possible direct endotoxin effect, no such correlations could be drawn for the calves of the present study. There was no evidence of appreciable pyrexia in either of the groups and, since O_2 consumption fell in a majority of the animals, increased basal metabolic rate cannot be stated to be the cause of increased Q. Similarly, acid–base status did not correlate with Q (Sahay, 1982). On the basis of these findings it is postulated that an increase in HR within reasonable limits coupled with lesser fall in CVP and MAP affected an increased Q in hyperdynamic shock and in the hyperdynamic phase of mixed shock.

TPR is believed to be inversely related to Q. Increased TPR has been observed in dog and man with decreasing Q (Perbellini et al., 1978; Shoemaker et al., 1970) and vice versa. On the contrary, in the present study, decrease in TPR was a consistent feature of early and late/terminal stages in hypokinetic (7.5% and 26.2%), hyperkinetic (23.1%, 43.5%) and mixed shock (16.8%, 36.5%). However, decreased peripheral resistance in low flow state has been observed in calves (Tikoff et al., 1966) as well as in monkeys (Hinshaw et al., 1966) following endotoxin administration. Low Q with decrease in TPR was also observed in buffalo calves following haemorrhagic shock despite increased catecholamine levels (Mirakhur et al., 1982). It is probable that in bovines, though catecholamines are released in response to stress, their effect is masked by vasodilating substances. Further, vascular changes in the individual organs might also be different and these might occur in opposite direction in the individual organs but with overwhelming effect of vasodilation (Hinshaw et al., 1966). Direct measurement of vascular resistance of individual organs may provide explicit explanation in this regard.

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REPRODUCTIVE DISORDERS CAUSED BY TRICHOTHECENE MYCOTOXINS IN A LARGE-SCALE PIG HERD

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In a large-scale pig herd, infertility occurred in nearly 40% of both the gilts and the pluriparous sows in a certain year. Trichothecene mycotoxins (T-2, HT-2) were detected in their feedstuffs in quantities of 1–2 mg/kg of feed on several occasions during this period. The detection of these mycotoxins in the feed coincided with the period of infertility.

Pathological, histopathological and ultrastructural investigations revealed multiplex cystic degeneration of the ovary, some signs indicative of a prolonged absence of ovulation, and the atrophy and lack of activity of the uterus and endometrium. Other histological findings included hypertrophy of the zona fasciculata in the cortex of the adrenal gland and hyperfunction of the thyroid gland. No pathological changes were found in other organs.

The same organs of healthy sows of 80, 110, 140, 180, 195, 210 days of age or older served as control.

Keywords. Reproductive disorder, pig, trichothecene, mycotoxin.

The fusariotoxin zearalenone acts primarily on the reproductive organs. First it was found to induce oedematous swelling of the vulva, vagina, uterus and nipples (oestrogen syndrome) in female animals. Later zearalenone was shown to cause also histopathological changes in the endometrium and ovaries, lesions which may impair reproductive activity. This effect of zearalenone has been studied extensively by Hungarian investigators (Palyusik, 1973; Ványi et al., 1973, 1974). Zearalenone-induced damages of the reproductive organs have been proved experimentally to occur also in male animals (Ványi and Széky, 1980). With the exception of the domestic hen *(Gallus domesticus)*, all domestic animal species, and also man, were found to be sensitive to this toxin (Ványi and Romváry, 1974). The effects of trichothecene fusariotoxins on the reproductive organs are less known.

Experimental evidence and observations made in field cases indicate that trichothecene toxins cause a significant drop in the egg production of different poultry species (chicken, goose, duck) (Ványi et al., 1980). The toxin and its toxic metabolite can be found also in the egg and may cause infertility or embryonic death during incubation (Ványi and Gajdács, 1983). The mechanism of the effect exerted on egg production is obscure. Hungarian authors (Palyusik and Koplik-Kovács, 1975) observed an atrophy of the ovary and oviduct in geese.

Volintir et al. (1971) established a causal relationship between field cases of infertility in brood-sows and the feeding of feedstuffs contaminated

with the fungus *Fusarium sporotrichioides*. The lesions appearing in the uterus and ovary of sows were studied by Hungarian and American authors (Palyusik et al., 1981). They found that T–2 toxin, as opposed to zearalenone, induced marked atrophy of both the ovaries and the uterus. The two toxins of different chemical structure partly reduce (oestrogen symptoms), partly enhance (damage of the ovary) the biological effect of each other.

Materials and methods

Detection of trichothecene mycotoxins

The feed of gilts and sows, and the dried corn and wheat used for its formulation were examined periodically. For the detection and identification of trichothecene fusariotoxins by thin-layer chromatography, the methods described by Stahr (1975) and Takitani et al. (1979) were used. Capillary gas chromatography was performed by using the simultaneous multimycotoxin detection method developed in our Institute (Ványi et al., 1981).

Histological examination

Samples were taken from 5 sows culled from the examined herd due to infertility, and from a total of 12 gilts given the same feeding (2 gilts each of 25, 35, 55, 65, 85 and 110 kg body mass were sampled). The gilts were killed by bleeding, and samples of appropriate size were taken from both ovaries, the oviduct, the uterine horn, the body of the uterus, the vagina and the adrenal and thyroid glands. The samples were fixed in 5% formalin and embedded in paraffin. For general examination, the sections were stained with haematoxylin and eosin. The reticular fibres of mucous membranes were stained with Azan's stain, while the secretory activity of their glands was followed by the periodic acid–Schiff (PAS) and the aldehyde–bisulfide–toluidine blue (ABT) reactions and examination under polarization microscope following the latter. The lipid content of the adrenal cortex was demonstrated in frozen sections by Fettrot staining.

As controls, 72 healthy gilts of different age and body mass (35, 55, 85 and 105 kg), derived from another stock, were bled and their appropriate organs were examined by the same methods (Glávits et al., 1983).

Electron microscopy

Samples were taken from both ovaries and the mucosa of the uterine horn of two infertile sows and of two gilts weighing 110 kg. The tissue samples were fixed first in 2.5% glutaraldehyde buffered with sodium cacodylate and

then in 1% osmium tetroxide. After embedded in Durcupan resin, the sections prepared with an OMU–3 "Reichert" ultramicrotome were post-stained with uranyl acetate and lead citrate, and examined in a Philips–201 CS electron microscope. As controls, samples taken from the same organs of eight gilts of 35, 55, 85 and 105 kg body mass (two in each category) were examined (Glávits et al., 1983).

Results

In a large-scale pig herd, 40% infertility of sows occurred in two successive years. During this period, trichothecene mycotoxins (T–2, HT–2) were detected in the sow feed in quantities of 1 to 2 mg/kg feed on several occasions. The detectability of these mycotoxins ran parallel with the period of infertility.

By *histological examination*, both ovaries and the mucous membranes of the inner genital tract showed similar histological picture in the 5 sows and in the 2 gilts weighing 110 kg. As opposed to the ovary of controls (Fig. 1), in these animals large numbers of tertiary follicles were seen distributed in the whole ovarian cortex. These follicles were of the same diameter, only slightly protruded over the surface of the organ, and all of them consistently showed signs of more or less severe atresia. Ova contained by these follicles showed

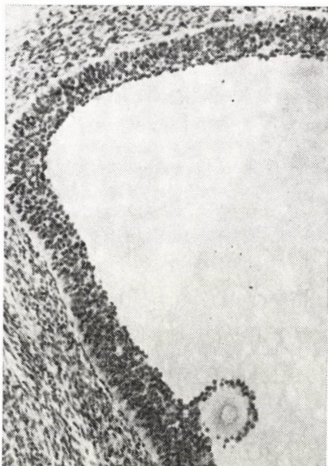


Fig. 1. Tertiary follicle in the ovary of a 190 days old, healthy gilt weighing 85 kg. Haematoxylin–eosin (H–E.), approx. ×160

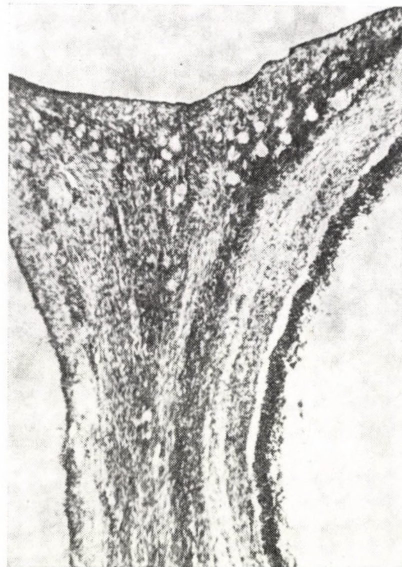


Fig. 2. Ovary of a sow given mycotoxin-containing feed for a long period: tertiary follicles showing cystic degeneration and compressing the ovarian stroma. H–E., approx. ×90

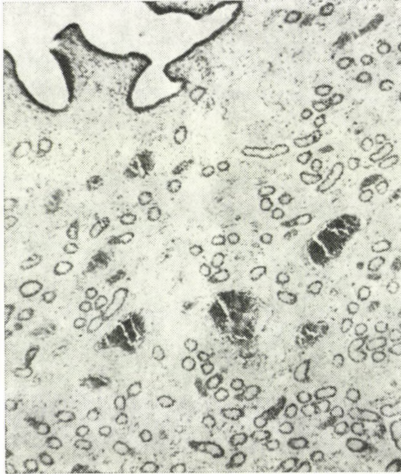


Fig. 3. Secretory-phase uterine wall of a 210 days old, healthy gilt weighing 105 kg. The glandular substance of the wide endometrium is well-developed and shows marked ramification. H–E., approx. ×25

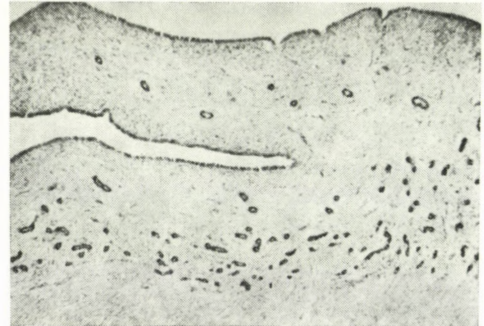


Fig. 4. Uterine wall of a sow given mycotoxin-containing feed for a long period: the narrow endometrium forms projections, the glandular substance is underdeveloped and shows little ramification. H–E., approx. ×25

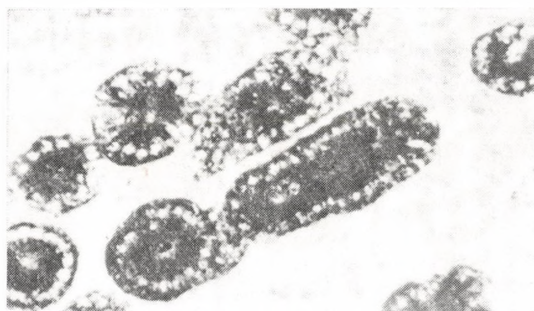
degeneration (pycnosis or lysis), while in the granulosa cell layer degeneration and, frequently, detachment from the hyaline membrane were seen (Fig. 2); the theca interna and the theca externa were generally intact. The ovarian stroma between these follicles had shrunk to narrow stripes, and the majority of primary and secondary follicles embedded in it had become atrophied. No forms indicative of ovulation (corpora haemorrhagica or lutea) were seen, the ovary of only one sow contained regressed corpora lutea (corpora albicantia). On the other hand, the ovaries of the control gilts of lower body mass showed signs indicating physiological maturation and atresia of follicles. In addition to these, corpora lutea indicative of ovulation were also seen in a part of the gilts weighing 105 kg (17 gilts).

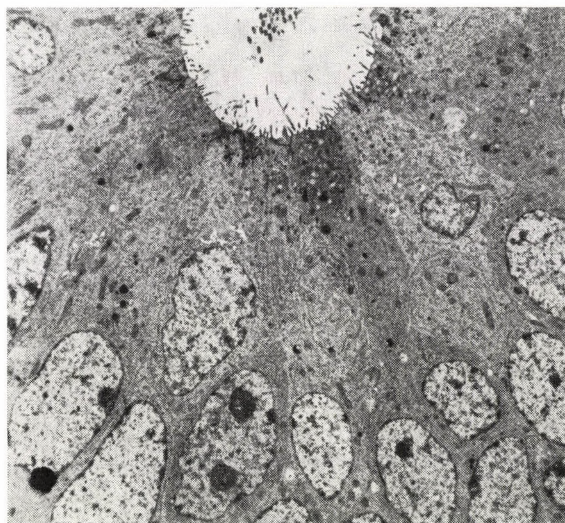
As compared with those of the controls (Fig. 3), the mucous membranes of the inner genital tract (oviducts, uterine horns, the body of the uterus) thinned down (Fig. 4) and showed no signs of hyperaemia or oedema. Cells of the surface epithelium of the endometrium had one or two nuclei, the epithelium was PAS- and ABT-negative, and frequently contained degenerated and expulsed epithelial cells. The glandular substance was atrophied and did not show ramification. As compared with the controls (Figs 5 and 6), the endpieces of glands were narrow, cells of the glandular epithelium were low, PAS- and ABT-negative, their cytoplasms contained few mitochondria, and their tubulo-vesicular system was atrophied (Figs 7 and 8). The stratified flattened epithelium of the uterine cervix and vagina consisted of two to five cell layers.

In these animals, the zona fasciculata of the adrenal cortex had widened and had become slightly deficient in lipids, as compared with the controls.

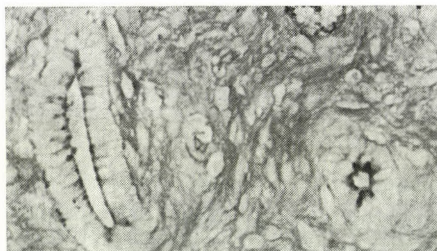
In the thyroid, the diameter of a considerable part (40 to 60%) of the acini had become smaller than the acini in the control thyroid (Fig. 9). The lining epithelium showed hypertrophy, transformation into columnar epithelium, and hyperplasia [formation of multilayered epithelium sometimes completely filling the acinus (Fig. 10)].

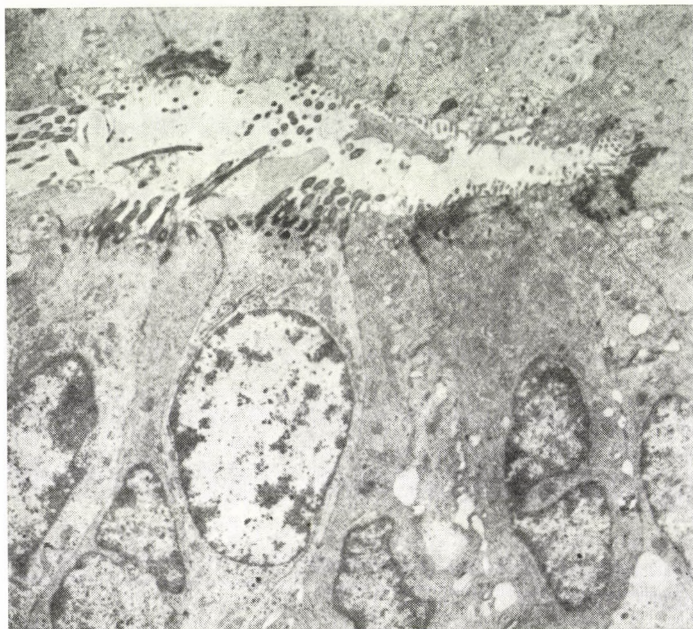
Chemical analysis of the feed demonstrated varying quantities of different trichothecene mycotoxins (T–2, HT–2 and occasionally diacetylnivalenole). The total quantity of these mycotoxins amounted to 1 to 2 mg/kg of feed. Analysis of the feed components has revealed that wheat was the component





Figs 5 and 6. Secretory-phase uterine wall of a 210 days old, healthy gilt weighing 105 kg: in the endometrium, the glandular epithelial cells with high cytoplasm show signs of intensive functioning. Periodic acid–Schiff (PAS) reaction, approx. ×400 (Fig. 5); Electron micrograph ×4100 (Fig. 6)





Figs 7 and 8. Uterine wall of a sow given mycotoxin-containing feed for a long period. In the endometrium, the glandular epithelial cells with low cytoplasm do not show signs indicative of functioning. PAS reaction, approx. ×400 (Fig. 7); Electron micrograph, approx. ×6400 (Fig. 8)

that contained the *Fusarium* toxins. The wheat content of the piglet diet was 15%, while that of the gilt feed I–III and the sow feed 10%. Attempts to demonstrate zearalenone failed.

Discussion

On the basis of the present results, we can state that the trichothecene fusariotoxins (T–2, HT–2) have an adverse effect on reproductive processes, as it was observed also by others (Volintir et al., 1971; Palyusik et al., 1981). When animals consume these toxins for a long time in a concentration of 1 to 2

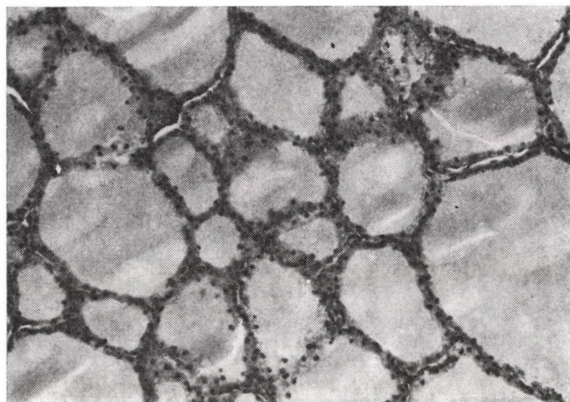


Fig. 9. Thyroid of a 210 days old, healthy gilt weighing 105 kg: medium and large acini lined by one-layered cuboidal epithelium. H–E., approx. ×160

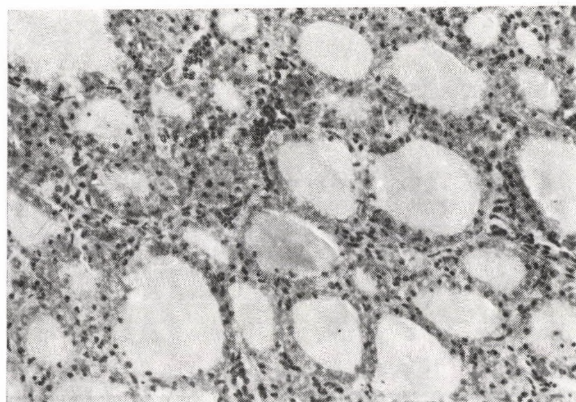


Fig. 10. Thyroid of a sow given mycotoxin-containing feed for a long period: small acini lined by high, occasionally multilayered, columnar epithelial cells. H–E., approx. ×160

mg/kg of feed, these adverse effects include cystic degeneration of the ovary (and thereby the inhibition of ovulation) and a consequent atrophy and loss of functional activity of the uterus, primarily the endometrium.

Both types of fusariotoxins (zearalenone and trichothecene toxins) cause disturbances in the hormonal balance of the organism. While zearalenone, due to its oestrogenic effect, induces proliferation of the mucous membranes of the inner genital organs without regard to the physiological status of the ovarian cycle, and thereby it necessarily causes an asynchronism in the function of ovaries and mucous membranes (Ványi et al., 1974), our experiences suggest that trichothecene toxins block the ovarian cycle due to their inhibitory effect on ovulation. This effect of the trichothecene toxins, in addition to their

other direct actions, prevented the rhythmic stimulatory effects of the follicular and corpus luteum hormones produced in the ovary from being manifested. In the present case, all these were expressed also in morphological and histochemical changes.

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STACHYBOTRYOTOXICOSIS AS A PREDISPOSING FACTOR OF OVINE SYSTEMIC PASTEURELLOSIS

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In December 1981, mass disease occurrence accompanied by sudden death was observed in a sheep flock of 970, 6 to 7 months old lambs in Northern Hungary.

Gross pathological findings included haemorrhages of varying size dispersed all over the body, mucosal haemorrhages and erosions in the intestinal tract, small greyish-white foci in the liver, and loss of fleece. *Pasteurella haemolytica* serotype-3 biotype-T (T3) strain was isolated from the parenchymal organs of 4 of the 5 lamb carcases examined.

The wheat straw used for both littering and feeding of lambs was strongly contaminated by the mould *Stachybotrys atra*. From samples of the mouldy wheat straw, macrocyclic trichothecene stachybotryotoxins (satratoxins G and H) were demonstrated by thin-layer chromatography and by the brine shrimp test. The identity of the toxins was verified by their transesterification into verrucarol.

Simultaneous occurrence of stachybotryotoxicosis and pasteurellosis was diagnosed in the lamb flock. Since the feed was changed immediately after the autopsy of carcases, and the use of the mouldy straw for feeding and bedding was stopped, only 41 lambs died.

In the authors' opinion, the development of acute systemic pasteurellosis in lambs that had harboured *P. haemolytica* strains of biotype T in their tonsils without showing symptoms was due to the predisposing effect of immunosuppressive stachybotryotoxins which can cause epithelial (mucosal) damage. The observations reported in the present paper are of interest from the viewpoint of differential diagnosis and furnish new knowledge of the pathogenesis of acute systemic pasteurellosis.

Keywords. Mycotoxin, *Stachybotrys atra*, *Pasteurella haemolytica*, predisposing factor, sheep.

The veterinary importance of subclinical mycotoxicoses lies in the fact that they predispose to different infectious diseases by decreasing the natural resistance and impairing the immune system of animals (Pier et al., 1980).

In Hungary, stachybotryotoxicosis is one of the most important mycotoxicoses in horses, sheep and cattle (Dankó and Tanyi, 1968; Dankó, 1976; Harrach and Bata, 1982), and occasionally it occurs also in man (Andrássy et al., 1980).

The fungus *Stachybotrys atra* (syn.: *S. alternans)* growing extensively on cellulose-rich forages (straw, hay) produces different, so-called macrocyclic trichothecene-type, mycotoxins (satratoxins G and H, verrucarin J, etc.) under both laboratory and natural conditions (Eppley, 1977; Harrach et al., 1981; Harrach and Bata, 1982). The above mycotoxins are, as a rule, immunosuppressive, and damage mucous membranes (Boján et al., 1976; Dankó, 1976). The cytotoxicity of the trichothecene-type mycotoxins is based on their

pronounced inhibitory effect on the protein and DNA synthesis of cells (Ueno, 1977).

On the basis of their colony morphology, biochemical properties and sensitivity to antibiotics, *Pasteurella haemolytica* strains fall into two biotypes (A and T), and by the indirect haemagglutination (IHA) test, they can be divided into 15 serotypes (Smith, 1961; Pegram et al., 1979; Fraser et al., 1982*b*). Biotype-T (serotype 3, 4, 10, 15) strains account for the so-called acute systemic pasteurellosis of growing, 5–12 months old lambs (Gilmour, 1980; Dyson et al., 1981; Fraser et al., 1982*a*). According to Dyson et al. (1981) and Angus and Gilmour (1981), this condition is not a true septicaemia, but can be regarded as a consequence of thromboembolism starting from the throat and upper alimentary tract as primary foci. Due to the effect of predisposing factors not yet known satisfactorily, the epiphyte *P. haemolytica* bacteria of biotype T normally present in the tonsils of healthy adult sheep multiply rapidly, and disperse to the different parenchymal organs through the lympho-haematogenic route (Gilmour et al., 1974; Gilmour, 1980; Dyson et al., 1981).

In the present paper, the relationship of stachybotryotoxicosis and acute systemic pasteurellosis is reported, since this can be important in the pathogenesis of pasteurellosis and from the differential diagnostic point of view.

Materials and methods

Post-mortem examinations. In December 1981, with an interval of 2 days, 5 lamb carcases were submitted for routine laboratory examination from a sheep flock of Northern Hungary, comprising 970 6 to 7 months old lambs of the Hungarian merino breed. The carcases showed a good body condition. Their external and internal gross examination was performed in the usual way.

Histopathology. Small organ samples were taken from the brain (brain stem, cerebellum, cerebral hemisphere) of all 5 carcases, and from the liver of 2. The samples were fixed in 10% neutral formalin. Frozen and paraffin sections were stained with haematoxylin and eosin, and by the Gram and Giemsa staining techniques.

Bacteriological examinations. Spleen and liver samples from all 5 carcases were inoculated onto blood agar plates with 8% defibrinated sheep blood, and bromothymol blue–lactose–crystal violet agar, while brain samples on blood agar plates only. The cultures were incubated under aerobic conditions at 37 °C for 24 h.

Biotyping and serotyping of P. haemolytica. P. haemolytica strains isolated from the spleen and liver of carcases were identified on the basis of their morphological and biochemical properties (Smith, 1961; Biberstein, 1978). Sero-

logical examinations were performed by using the IHA test (Biberstein, 1978). Antisera to serotypes 1–15 were produced in rabbits.

Mycology. For the isolation of *S. atra* strains, black-spotted "suspect" stalks of straw collected from wheat straw stored in the stack used for feeding of lambs and also for bedding, were placed into Petri dishes, on filter-paper soaked with van Iterson's solution. The stalks were incubated at 22–24 °C for 7 days, and the subsequent identification was performed according to Szigeti (1971).

Toxin analysis. Demonstration of trichothecene-type stachybotryotoxins in wheat straw samples by thin-layer chromatography and the verification of their identity by transesterification to verrucarol were performed as described earlier (Harrach and Bata, 1983). The toxicity of the isolated mycotoxins was checked in the brine shrimp test (Harrach et al., 1981).

Results

History and clinical findings. In the above lamb flock feeding of, and bedding with, baled wheat straw which had got wet at the time of harvesting, in August 1981, was started at the end of November of the same year. Since that time the baled straw had been stored in stacks on the farm. In addition to wheat straw, the lambs were fed dried grain corn and beetroot tail, feed that they had received also earlier. The first cases occurred approximately two weeks after the beginning of straw feeding, and the symptoms included a short-term listlessness, inappetence, strong salivation and weakness. Some

Fig. 1. Loss of fleece in an approximately 7 months old lamb diseased in stachybotryotoxicosis

animals exhibited also dyspnoea, foamy and, occasionally, blood-streaked nasal discharge and diarrhoea. Part of the animals were febrile (with a body temperature around 41 °C). Some of the affected animals responded to antibiotic therapy (penicillin + streptomycin i. m.), but most of them died within 24–48 h. A striking feature of the condition was that the fleece of the lambs could be pulled out in clumps or it hung down in tufts. Lambs with fleece falling out were seen in the flock even after the beginning of January 1982, after deaths had ceased to occur (Fig. 1).

Pathological findings. Of the lambs submitted a few hours after death, the fleece of 3 was hanging down in tufts or could be pulled out easily. There were flat, expanding ecchymoses of varying size in the subcutaneous connective tissue all over the body. The regional lymph nodes were swollen, dark red and succulent. Petechiae and ecchymoses were seen under the serous membranes of the abdominal cavity, in the diaphragm, under the capsule of spleen and under the pleura. The spleens were normal in shape and size. In two cases, the liver was found to contain numerous small, greyish-white necrotic foci, while the other 3 livers showed degeneration. The mucous membranes of the rumen and abomasum contained haemorrhages and erosions of varying size and having indistinct margins, while that of the small intestine had minute, scattered haemorrhages or, in other places, those showing a "sprinkled" character. In one of the carcases, the small intestinal mucosa showed also small erosions and haemorrhagic-diphtheroid sloughs. The kidneys were pale brown and succulent. The mucosa of the throat was hyperaemic, swollen, and in two cases the tonsils contained yellowish-white or greyish-yellow, crateriform, deep necrotic foci. The hard palate of one lamb exhibited small greyish-yellow necrotic areas, while in the other carcases the oral mucosa was intact. The presence of foamy, reddish contents in the trachea and nasal passages indicated expressed pulmonary oedema and hyperaemia. Petechiae were seen under the epicardium and endocardium. Parasites were not seen in the brain and in the nasal passages with the unaided eye. The nasal mucosa was dark red and was covered with reddish, foamy secretion.

Histopathological findings. In brain sections stained with haematoxylin and eosin, changes indicative of inflammation were seen neither in the brain nor in the meninges. In the liver of two carcases, necrotic foci of varying size containing clumps of bacteria were observed (Fig. 2). The bacteria present in the clumps proved to be Gram-negative. In places, the necrotic area extended to several neighbouring hepatic lobules (Fig. 3). No regularities were found to exist in the localization of the necrotic foci.

Bacteriological findings. In cultures from the spleen and liver of 4 carcases, beta-haemolysing, round colonies 1–1.5 mm in diameter, having a darker centre grew out in pure culture. The bacterium was identified as *P. haemolytica* biotype-T, serotype-3 (T3) strain. Bacteriological examination of the spleen

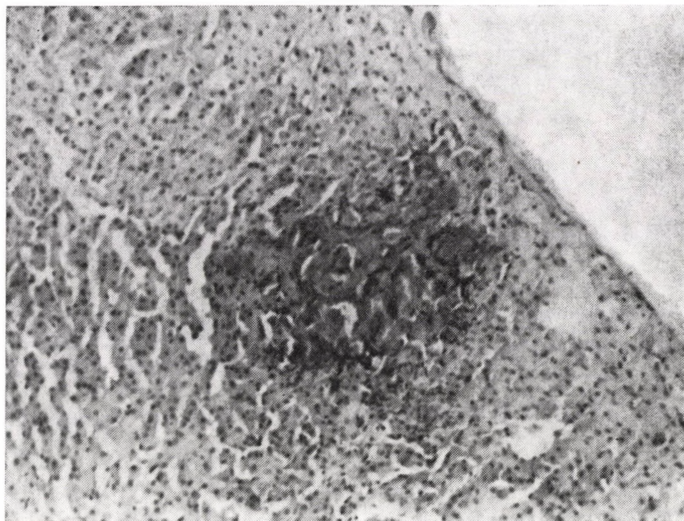

Fig. 2. Circumscribed necrotic focus in the liver with clumps of bacteria. H. and E., approximately ×65

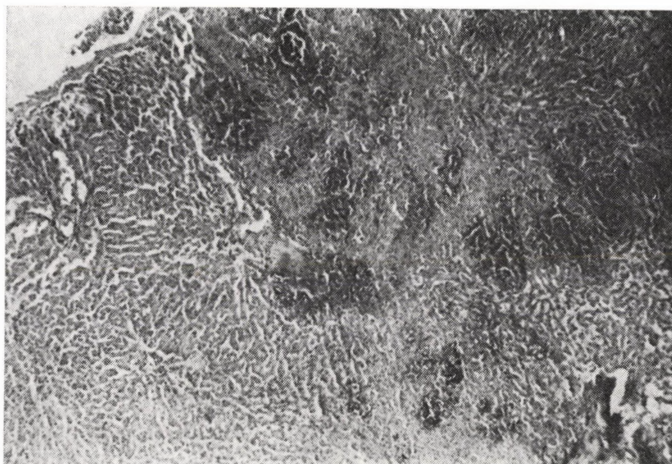

Fig. 3. Necrotic area extending to several neighbouring lobules of the liver. H. and E., approximately ×25

and liver of the fifth carcase, and that of the brain of all 5 carcases gave negative results.

Mycological and mycotoxicological findings. From samples of the black-spotted wheat straw, a mould identifiable as *Stachybotrys atra* was isolated in pure culture. Analytical studies revealed the presence of *satratoxin G* and *H* macrocyclic trichothecene stachybotryotoxins. Their identity was proved by their transesterification into *verrucarol.* These *Stachybotrys* toxins had already

been isolated in Hungary, viz., from mouldy bedding straw in connection with stachybotryotoxicosis of calves (Harrach and Bata, 1982), and they are produced by the type strain (ATCC 34915) isolated in Hungary from fatal cases observed among horses (Harrach et al., 1981). The mycotoxins isolated by us have proved to be toxic in the brine shrimp test, and belong to the group of trichothecene mycotoxins considered most toxic, i.e. to Ueno's group D (1977).

Discussion

Simultaneous occurrence of stachybotryotoxicosis and acute systemic pasteurellosis was diagnosed in the lamb flock under study on the basis of the field investigations and laboratory examinations. The clinical symptoms and pathological lesions resembled those described for both stachybotryotoxicosis of sheep (Dankó, 1976; Schneider et al., 1979) and the disease caused by biotype-T *Pasteurella haemolytica* in growing lambs (Ratalics and Szabó, 1964; Gilmour, 1980; Dyson et al., 1981). T3-type *P. haemolytica* strains were isolated in pure culture from the parenchymal organs of 4 of the 5 lamb carcases examined by us. In two cases, the small necrotic foci present in the liver (Figs 1 and 2) contained *P. haemolytica* in the form of bacterial emboli.

P. haemolytica strains have been isolated from the organs of sheep succumbed to "stachybotryotoxicosis" also by Dankó (1976) and Schneider et al. (1979); however, the latter authors did not type their isolates. In agreement with the observation of Ratalics and Szabó (1964), the disease seen by us had not ceased to occur until the feed of lambs was changed.

The presence of macrocyclic trichothecene stachybotryotoxins in samples of the wheat straw used for bedding and feeding was verified also by chemical analysis. The death of the bacteriologically negative lamb examined by us, and that of lambs died in the sheep farm within a short time without a febrile response in a condition characterized by haemorrhages all over the body must have been due solely to stachybotryotoxicosis. Pathological lesions were more expressed in the carcases the organs of which yielded *P. haemolytica* upon isolation. Earlier Dankó (1976) made a similar observation in experimental stachybotryotoxicosis of sheep.

In our opinion, the *P. haemolytica* T-biotypes present in the throat as epiphyte bacteria became activated under the influence of the immunosuppressive stachybotryotoxins having a damaging effect on mucous membranes. The activated bacteria dispersed to the different parenchymal organs through the lympho-haematogenic route. Thus, the effect exerted by mycotoxins may be one of the important factors in the pathogenesis of acute systemic pasteurellosis.

The pathology of acute systemic pasteurellosis resembles that of the so-called pyosepticaemia of horses, a disease caused by *Actinobacillus equuli*

(Széky, 1962; Dankó and Tanyi, 1968). In Hungary, pyosepticaemia often occurs simultaneously with stachybotryotoxicosis, as a complication of the latter (Dankó and Tanyi, 1968). In horses, a similar predisposing role of T-2 toxin, another trichothecene mycotoxin produced by *Fusarium* species, has already been substantiated (Szigeti et al., 1977).

When diagnosing acute systemic pasteurellosis in lambs, the predisposing role of feeding mouldy feed contaminated mainly by *Stachybotrys*, *Fusarium* and *Myrothecium* spp. (Ueno, 1977) and containing trichothecene-type mycotoxins should be born in mind also in other parts of the world. Elucidation of the relationship of mycotoxicosis and pasteurellosis is indispensable in reducing losses and in the prevention.

In our case, owing to the immediate change of feed following the necropsies (presumptive diagnosis: stachybotryotoxicosis), and probably also to the low number of animals harbouring *P. haemolytica* T-biotypes, only 41 lambs died in the course of about 3 weeks.

As opposed to that obtained for Great Britain (Fraser et al., 1982*a*), in Hungary the rate of *P. haemolytica* T-biotypes isolated from necropsy material is low (Fodor et al., 1984).

In the development of infectious diseases, subclinical mycotoxicoses are none the less important. Under field conditions, the verification of their predisposing role is difficult, since the symptoms and lesions of the accompanying infectious diseases usually conceal those of mycotoxicoses (Pier et al., 1980).

In the sheep flock studied by us, about 1 month after the case reported above, during silage feeding, encephalitis caused by virulent *Listeria monocytogenes* strains of serotype 1/2 occurred in masses in 7–8 months old lambs of impaired immune system.

Dankó (1976) fed stachybotryotoxin-containing feed to pregnant ewes in an advanced stage of pregnancy. Although the ewes did not abort during the period of experiment, their live-born lambs showed very low leucocyte counts, indicative of in utero impairment of the immune system. Under field conditions and in certain flocks, this impairment can be of decisive importance in suckling lamb losses, and probably also in the magnitude of losses due to septicaemia and pleuropneumonia caused by *P. haemolytica* A-biotypes.

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METABOLIZATION OF TRICHOTHECENE TOXINS (T-2 TOXIN AND DIACETOXYSCIRPENOL) IN EMBRYONATED HEN'S EGG

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In parallel experiments trichothecene toxins (T-2 toxin and diacetoxyscirpenol, 5 μg/egg) were injected into the yolk of embryonated hen's egg and incubated for ten days. The metabolites formed during incubation were analysed by a capillary gas chromatographic method. They were hydrolytic products of T-2 toxin and diacetoxyscirpenol, resulting in T-2 tetraol and scirpentriol, respectively.

Keywords. Biotransformation, trichothecenes.

Mycotoxin-producing mold species may produce different kinds and amounts of toxins. After the consumption of feedstuffs containing mycotoxins, toxic metabolites poorly known in nature may be formed in the animal organism.

In vitro conversion of T-2 toxin into HT-2 toxin was observed after incubation of T-2 toxin with $9000\times g$ supernatant fluid of human and bovine liver homogenates (Ellison and Kotsonis, 1974). Robinson and Mirocha (1976) reported that T-2 toxin was converted into neosolaniol, HT-2 toxin and T-2 tetraol toxin. Matsumoto et al. (1978) examined the fate and distribution of T-2 toxin in mice with tritium-labelled T-2 toxin. Chi et al. (1978) reported that T-2 toxin was metabolized in one-day-old broiler chicks into HT-2 toxin, neosolaniol, deacetyl-HT-2 toxin and T-2 tetraol toxin.

This report deals with the transformation of T-2 toxin and diacetoxyscirpenol (DAS) in embryonated hen's egg and with the rate of metabolization.

Materials and methods

Toxin production for analytical standard

To produce T-2 toxin, HT-2 toxin and neosolaniol, *Fusarium 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 N 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.

Experiments with hen's eggs

T-2 toxin and DAS were dissolved in ethanol (1 μg/μl). Five μl of toxin solution was injected into the yolk of embryonated hen's eggs. Twenty eggs were tested for each toxin. They were incubated at 40 °C for 10 days. Two eggs were used for each analysis.

Chemical analysis

Two eggs were admixed with 100 ml of acetone and 5 g of $(NH_4)_2SO_4$. The mixture was filtered and evaporated under vacuum. To the residue 20 ml of methanol–water (4 : 1) was added and extracted three times with petroleum ether. The aqueous layer was dried with anhydrous Na_2SO_4 and evaporated

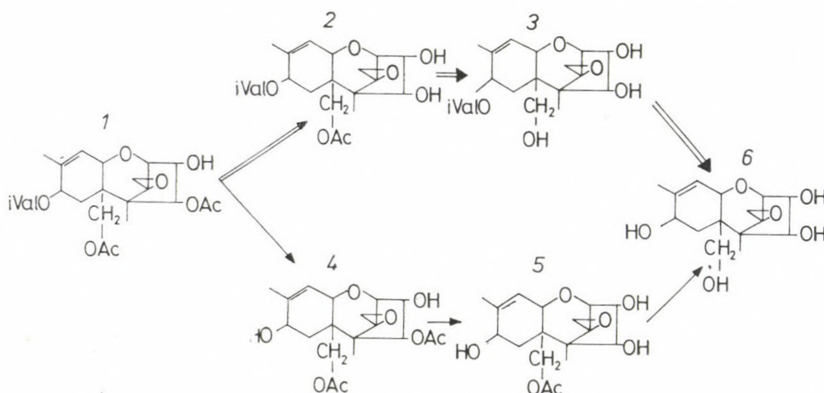

Fig. 1. Scheme of T-2 toxin metabolism (*1* T-2 toxin, *2* HT-2 toxin, *3* T-2 triol, *4* Neosolaniol, *5* Deacetyl-neosolaniol, *6* T-2 tetraol)

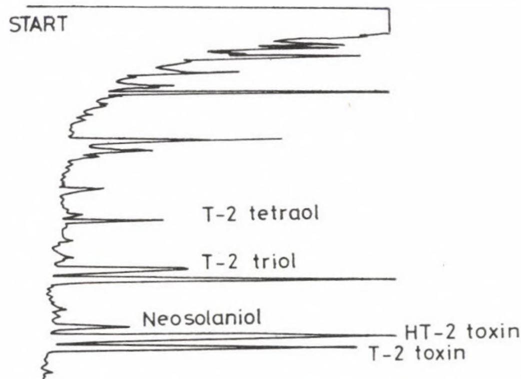

Fig. 2. Chromatogram of egg 2 days after injection with T-2 toxin

to dryness. The residue was purified on silica gel column. The sample was analysed by high-performance thin-layer chromatography (HPTLC) and after silylation by capillary gas chromatography (Bata et al., 1983).

Results and discussion

During ten days of incubation two eggs per day were investigated. When T-2 toxin was injected into the yolk, T-2 toxin, HT-2 toxin, neosolaniol, T-2 triol, T-2 tetraol and two further, still unidentified, metabolites thought to be trichothecenes were found. Figure 1 shows the scheme of T-2 toxin metabolism, and in Fig. 2 the chromatogram of an egg injected with T-2 toxin can be seen.

Table I

Biotransformation of T-2 toxin in embryonated hen's egg

Incubation time in days	T-2 toxin	HT-2 toxin	Neosolaniol	T-2 triol	T-2 tetraol
	μg per egg				
0	2.90	0	0	0	0
1	1.65	1.20	0.10	0	0
2	1.00	1.35	0.27	0.10	0.10
3	0	0.15	0.18	0.95	1.80
4	0.85	0.10	0.10	1.00	1.30
7	0	0.20	0.10	0.75	1.35
8	0	0	0.05	1.15	0.85
9	0	0	0	0.60	2.40
10	0	0	0	0.20	1.35
11	0	0	0	0	2.00

Table II

Biotransformation of DAS in embryonated hen's egg

Incubation time in days	DAS	Monoacetoxy-scirpenol	Scirpentriol
		μg per egg	
0	3.15	0	0
1*	2.90	0.05	0
1+	2.85	0.35	0
2*	2.65	0.80	0
2+	2.00	1.20	0.10
3*	1.25	2.10	0.40
3+	0.70	2.20	1.05
6	0.20	1.55	1.60
7	0.05	1.05	1.55
8	0	0.85	1.65

* processed a.m.; + processed p.m.

DAS, monoacetoxyscirpenol, scirpentriol and two other metabolites not identified so far (not identical with the two found in the eggs injected with T-2 toxin) were found in case of DAS injection.

The results are summarized in Tables I and II.

It can be seen from the results that both T-2 toxin and DAS transformed rapidly in the egg yolk and metabolites were formed. All the examined metabolites are toxic but less than T-2 toxin or DAS. The half-life of T-2 toxin was 48–60 h and in 8–9 days it hydrolysed to T-2 tetraol toxin. The half-life of DAS was only 36–48 h and in 7–8 days almost all metabolites converted to scirpentriol.

Probably both T-2 tetraol and scirpentriol decompose further in the egg yolk to unknown metabolites.

The inhibition of development of the chicken embryo was attributable to the trichothecenes.

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EFFICIENCY CONTROL OF EMBRYO TRANSFER BY PROGESTERONE ANALYSIS IN RECIPIENT HEIFERS

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Plasma progesterone (PROG) concentration of 129 heifers, used as recipients for embryo transfer, was measured on the day of selection, at presumed time of oestrus, at the time of embryo implantation and at the time of possible return to oestrus (13 and 34 days later). On the day of selection all heifers were in the luteal phase of the cycle, as proved by rectal palpation. Pregnancy was confirmed by rectal examination on the 70th–90th days.

Of the animals, 50.4% conceived and calved, 25.6% received unsuccessful implantation, while in 24.0% early embryonic mortality has been confirmed.

There was a discrepancy between the result of rectal examination (presence of a corpus luteum) and the < 5 nmol/l (low) PROG level in 25.6% of cases. Only 30.3% of these heifers got pregnant, while in the "high" PROG group (>5.0 nmol/l) 57.3%. In the "high" PROG group 4.2% of the heifers failed to respond to $PGF_{2\alpha}$ synchronization, while in the "low" PROG group the percentage of non-respondent heifers was 45.4%.

At the time of embryo transfer, PROG level was <5.0 nmol/l in 57.6% of heifers of the "low" PROG group and in 35.4% of those in the "high" progesterone group.

It is suggested that knowledge of the progesterone profile of candidates for selection as recipients may significantly increase the efficiency of embryo transfer.

Keywords. Embryo transfer, cattle, efficiency control, progesterone analysis, recipient.

The use of embryo transfer as a zootechnical procedure is gaining ground rapidly (Fulka, 1982; Newcomb, 1982; Solti, 1981). This technique differs from the previous biotechnical methods not only in its novelty but also in its complexity and in the fact that it requires more cautious work to be carried out successfully. Since 1977 several Hungarian authors have reported their results obtained with embryo transfer in cattle and other animal species (Becze et al., 1979; Becze et al., 1977; Hahn et al., 1978; Haraszti and Rónay, 1977; Mészáros, 1982; Solti, 1983; Solti et al., 1978). One of the fundamental biological conditions of successful embryo transfer is the accurate coincidence of the cycle of donor and recipient animals. Therefore, animals in the luteal phase of the cycle are chosen as recipients, and they are synchronized by $PGF_{2\alpha}$ treatment in appropriate time. If the treatment is successful, oestrus will take place within 3 days. In our practice, embryo transfer is performed on the 7th or 8th day of the cycle.

Both the selection of recipients and the evaluation of the success of $PGF_{2\alpha}$ treatment can be performed by clinical methods, i.e. by rectal examination. However, errors cannot be ruled out by rectal examination, even if the

examiner has great experience. It seemed, therefore, justified to check the correctness of the clinical results by determination of plasma progesterone concentration. Determination of the plasma progesterone concentration at the time of possible returns to oestrus (on the 21st and 42nd days after oestrus, i.e. on the 13th and 34th days after transplantation) allows rapid and accurate evaluation of the efficiency of embryo transfers and detection of early embryonic losses. The present paper reports our experiences obtained with the above method. Our results are reported also in Hungarian, in the form of an authors' summary due to appear in Magyar Állatorvosok Lapja (Pethes et al., 1983).

Materials and methods

The experiments were conducted at the Embryo Transfer Station of the National Advisory Board for Animal Breeding and Nutrition (OTÁF), at Üllő. In 1980 and 1981, 129 1.5 to 2.5 years old Hungarian Fleckvieh heifers of average body condition were chosen as recipients for surgical embryo transfer.

By rectal examination, all animals were found to be in the luteal phase (days 6–14) of the cycle. On the day of selection for transfer the heifers were given $PGF_{2\alpha}$ (20 mg Enzaprost F inj. ad us. vet., Chinoin). The majority of animals began to show the symptoms of oestrus within 72 h. On the 10th day after treatment (the 8th day of the cycle), an 8 days old embryo was surgically implanted to the uterine horn ipsilateral with the corpus luteum. The success of implantation was checked by rectal examination performed 70–90 days afterwards.

For progesterone analysis, blood samples were taken from the jugular vein into heparinized tubes at the following sampling times: at selection for transfer (day –3), at the presumed time of oestrus (day 0), at the time of embryo implantation (day 8) and at the times of possible returns to oestrus (days 13 and 34 after embryo implantation, which correspond to days 21 and 42 of pregnancy). The samples were centrifuged on the spot, transported to the laboratory and stored at —20 °C until used.

The progesterone concentration was determined, after extraction with diethyl ether, according to Pérez (1979) by radioimmunoassay using antibody of 1 : 6000 dilution and designated as G-254-II. The measuring error within series was 5.7%, while the inter-series error was 9.7%. The binding percentage of antibody was 38%, while the sensitivity limit of the method 16 pmol/l (5 pg/ml).

Embryo transfer was considered unsuccessful if progesterone concentrations lower than 3.5 nmol/l, characteristic of non-conceived animals, were found on two subsequent occasions, and the rectal examination carried out between days 70 and 90 also gave a negative result, i.e. consistent with the

above progesterone concentration. Successful embryo transfer was indicated by progesterone concentrations higher than 4.0–4.5 nmol/l at the above times, and by a rectal finding which confirmed pregnancy. In cases when the first, or both, progesterone levels indicated successful transfer, but rectal palpation performed between days 70 and 90 failed to confirm pregnancy, death of the implanted embryo between days 21 and 42, or possibly after day 42, or an alteration of cycle length was postulated. Statistical evaluation was performed by Student's *t* test (progesterone concentration) and by the help of a so-called four-square table (frequency distribution).

Results

Of the 129 recipient animals, 65 (50.4%) conceived and calved (group I), 33 (25.6%) received unsuccessful implantation (group II), while in further 31 heifers (24.0%), in spite of a probably successful implantation, the transferred embryos died (group III). In 5 cases embryonic death occurred between days 21 and 42, while in 26 heifers after day 42. The results of progesterone assays are summarized in Fig. 1.

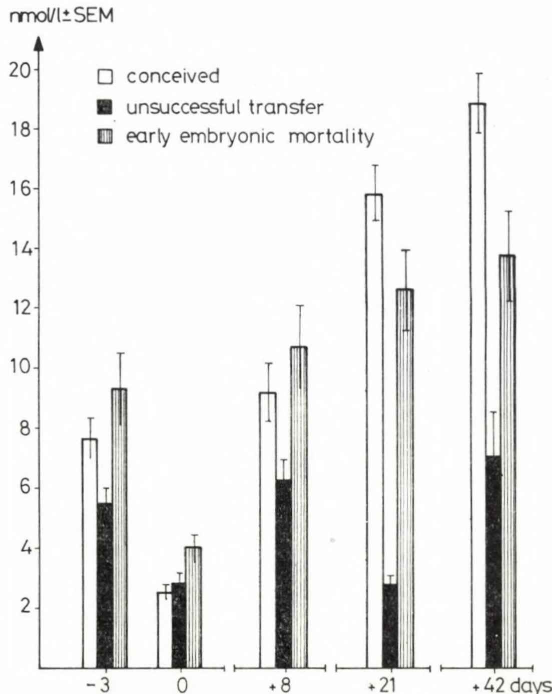

Fig. 1. **Plasma progesterone concentrations in recipient heifers**

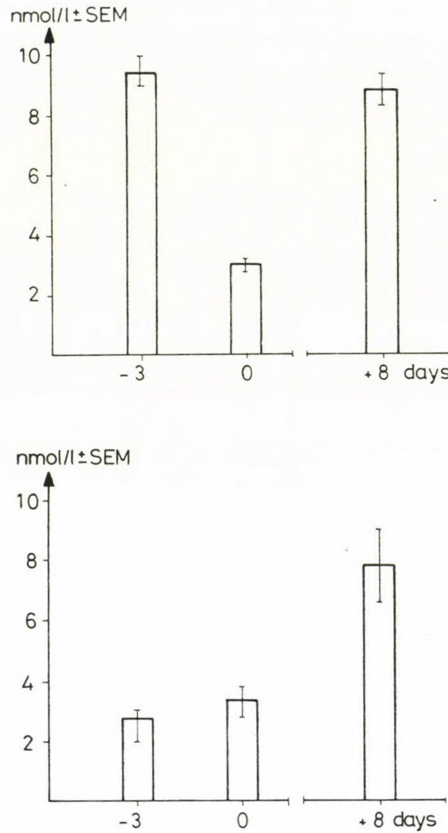

Fig. 2. a Correctly selected heifers (progesterone concentrations above 5.0 nmol/l); *b* incorrectly selected heifers (progesterone concentrations below 5.0 nmol/l)

In 33 of the 129 animals (25.6%), the clinical examination performed on the day of selecting for embryo transfer and the progesterone assay gave discrepant results: in such cases the plasma progesterone concentration was lower than 5.0 nmol/l in spite of the rectally palpated corpus luteum. Only 10 of these heifers became pregnant (30.3%), while in the remainder (69.7%) transplantation was unsuccessful or the embryo died after being implanted.

In the remaining 96 heifers (74.4%) of the 129, the progesterone concentration of the blood plasma (>5.0 nmol/l) agreed with the result of rectal examination. Of these 96 heifers, 55 (57.3%) conceived, the embryo died in 18 (18.7%), while the transplantation was unsuccessful in 23 (24.0%). Of the 33 animals regarded as incorrectly selected on the basis of the measured progesterone concentration, 15 (45.4%) failed to respond to $PGF_{2\alpha}$ and 19 (57.6%) had progesterone levels lower than the required minimum (5.0 nmol/l) on the day of transplantation.

Of the 96 heifers with plasma progesterone concentrations higher than 5.0 nmol/l on the day of selection, only 4 (4.2%) failed to respond to $PGF_{2\alpha}$, and 34 animals (35.4%) had lower than desirable plasma progesterone on the day of transfer. Plasma progesterone concentrations measured in the two groups of animals are shown in Fig. 2.

Discussion

The above results indicate that the determination of progesterone in the plasma of heifers receiving embryo transfer provides results utilizable in several ways.

It is striking that the heifers with a plasma progesterone concentration lower than desirable (about 5 nmol/l) on the day of synchronization respond to the injection of $PGF_{2\alpha}$ less frequently ($P < 0.01$) than the animals having a well-functioning corpus luteum. Subsequently, on the day of embryo transfer, nearly 60% of the heifers were in an endocrine status suboptimal or unsuitable for embryo implantation (plasma progesterone lower than 5 nmol/l). This was probably due to an inefficient synchronization. The difference between the two groups of heifers was also significant ($P < 0.05$) in respect of the proportion of animals with plasma progesterone concentrations above 5 nmol/l on the day of transfer. In part of the cases, the failure of synchronization was probably due to the insufficient $PGF_{2\alpha}$ doses applied (20 mg/animal); presumably, 25–30 mg doses would have resulted in better synchronization (Solti, 1983). Since, however, all animals received the same dose, the $PGF_{2\alpha}$ dose used cannot account for the difference observed between the two groups of heifers.

These facts explain why about 30% of the heifers receiving unsuccessful transfers and about 40% of those with embryonic mortality came from a group representing about one-fourth of the total number of animals, while only 30% of the conceived heifers belonged to this group. As regards the number of conceived animals, there was a significant ($P < 0.01$) difference between the two groups. Naturally, the high (>5.0 nmol/l) plasma progesterone concentration found on the day of synchronization and transplantation cannot in itself guarantee the success of transfer. However, on the basis of our results it is evident that by progesterone assay performed on the day of synchronization and, based on the results thereof, by exclusion of heifers with insufficient plasma progesterone concentrations from embryo transfer, the efficiency of transfer can be greatly increased.

Progesterone assay can play an important role also in the rapid and accurate evaluation of the efficiency of embryo transfer. On the 13th and 34th days after transplantation, there are highly significant ($P < 0.001$) differences

in plasma progesterone between conceived animals and those that have received an unsuccessful transfer. Recipient animals that have received unsuccessful embryo transfer and those bearing embryos died after implantation are reliably distinguishable on the ground of the plasma progesterone level. By clinical examination the latter can be distinguished only in case if they return to oestrus. Thus, plasma progesterone furnishes important, otherwise unobtainable, additional information on the efficiency of embryo transfer.

The present results suggest that the risk of embryonic death is highest in the period around the time of implantation (we qualified these recipient heifers as those having received unsuccessful transplantation). This is well indicated by the low progesterone concentration found on day 13 after implantation (i.e. day 21 of the cycle). Our experiences suggest that the probability of embryonic losses is relatively low between days 14 and 34 after implantation, while from day 34 to days 70–90 (i.e. up to the time of rectal examination) the risk of embryonic death is again higher.

Authors dealing with embryo transfer have studied the progesterone concentration primarily in donor animals (Solti et al., 1978), and there are only few data as regards the progesterone concentrations of recipient animals.

In their studies on milk progesterone levels, Hahn et al. (1977) observed great deviation in values measured on the 7th day after oestrus, and attributed this to the inadequacy of synchronization. Hasler et al. (1980) found significantly higher progesterone concentrations in blood samples taken on days 20–22 after embryo transfer from pregnant animals than in those obtained from non-pregnant ones. The higher progesterone levels found in animals with a shorter duration of oestrus (Hasler et al., 1980) suggest that the beta-carotene supply, which is related to the conception rate (Mészáros, 1982), may also have a role in the above phenomenon (Pethes et al., 1983).

Our results agree with those of Genazzani et al. (1980) who, based on a different sampling system, suggested that knowledge of the pre-synchronization plasma progesterone concentration promotes the selection of suitable recipients. Other authors (Remsen et al., 1982) concluded to the efficiency of embryo transfer from the progesterone concentration measured on the day of transfer. They reported a lower conception rate when the progesterone concentration was 2.0 ng/ml. According to similar observations made by the present authors, however, progesterone assay performed before synchronization is the method of choice under the conditions of Hungarian animal breeding practice, since this method gives reliable and utilizable results. The present data suggest that the efficiency of embryo transfer can be considerably improved by selecting animals with plasma progesterone concentrations higher than 5 nmol/l.

Acknowledgements

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GROWTH DYNAMICS OF THE PELVIC REGION IN CATTLE

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The analysis of growth tendencies of internal pelvic measurements in 78 Limousine × Red and White Hungarian females revealed several points of interest related to the understanding of the formation of the birth canal.

Of the three measurements evaluated here, interpsoadic distance and intertuberous distance are most correlated with each other but have different growth rates. When compared to each other, differential growth of these two measurements points to faster widening in the cranial section of the pelvis. External widths of the hindquarters in adult cows, on the other hand, display much more harmonic growth, thus biasing calculations aimed at the accurate estimation of internal structure using such easily measurable external dimensions.

Keywords. Growth dynamics, pelvis, cattle.

In addition to the genetical work involved in planning of crossing programmes, efforts must be made to minimize costs at all levels. One of the problems arising from the improvement of beef production is the contradiction between the size of dams and that of sires belonging to large-stature breeds with superior beef yield. In extreme situations, calving difficulties may be the source of a significant economic loss and thus are of major concern to veterinarians. The determination of pelvic dimensions using external measurements of the hindquarters has largely been unsuccessful. This paper is an attempt to at least provide a partial explanation of this problem, using a sample each of external and internal pelvic measurements.

Materials and methods

Although problematic calving is not the case with the offspring produced by the beef-purpose Limousine breed and the dual-purpose, Fleckvieh-type Red and White Hungarian, the main body of data in this study comes from F_1 and R_1 females of this crossing. Both generations are based on the use of frozen semen from Limousine sires. In a previous study (Bartosiewicz and Gere, 1983) the mean values for the three main internal measurements did not show significant differences at a $P \leq 0.05$ level. These three measurements, the median diameter (conjugata vera), interpsoadic distance (diameter transversa aperturae pelvis intermediae) and intertuberous distance (diameter trans-

versa aperturae pelvis caudalis) were used again in this study and plotted against each other in logarithmic bivariate plots. This technique is widely used in producing simple linear equations readily available for easy interpretation of the relative growth process (Fábián, 1959; Gere and Bartosiewicz, 1979*a*; Bartosiewicz, 1981, etc.). Slopes of the functions obtained this way are the graphic representation of the growth coefficients which show equal growth intensity for the independent and dependent variables when tangent α equals 1 (Brody, 1927; Fábián, 1969).

The three linear anatomical parameters were measured in vivo on 78 animals at the Nagykőrös State Farm and provided the raw data for testing the following alternative hypotheses:

H_0: There is no difference between the growth dynamics of internal pelvic measurements in the studied population.

H_r: The proportions defined by these measurements are constant, but result from the differential growth of internal pelvic dimensions.

The results of calculations should not be accepted unless confirmed by a significant correlation (Guilford, 1956) at a $P \leq 0.05$ level.

Results

Growth tendencies of the internal pelvic measurements are characterized by the allometric equations shown in Table I. Considering that only a low, but significant, correlation seems to exist between each pair of variables, we paid major attention only to the relationship between the two width measurements (interpsoadic distance and intertuberous distance) marked by the highest

Table I

Allometric equations describing the interrelationships between the three internal pelvic measurements and univariate statistics of the input data
(all measurements in centimeters)

Dependent variables (y)	Independent variables (x)			
	lg (median diameter)		lg (interpsoadic distance)	
	Growth coeff.	*r*	Growth coeff.	*r*
lg (interpsoadic distance)	$y = 0.7658+0.2885x$	0.2616*		
lg (intertuberous distance)	$y = 0.7727+0.2886x$	0.2475*	$y = 0.6606+0.4184x$	0.3958***

	Univariate statistics (n = 78)		
	$\bar{x}$	*sd*	*cv*
Median diameter	17.3820	1.0562	0.0608
Interpsoadic distance	13.2923	1.0622	0.0799
Intertuberous distance	14.0276	1.0659	0.0760

coefficient of correlation which is also significant at a $P \leq 0.001$ level, far beyond the value required by the test implications. In this case one can reasonably state that the coefficient of growth is indicative of a relatively slow widening in the caudal pelvic region characterized by the intertuberous distance.

Discussion

In order to properly view the relationship between the two width measurements established in Table I, the results were placed in a wider context. External hindquarter measurements of 78 Limousine × Hungarian Fleckvieh cows were used to provide a background to this study.

When rump width (measured between the pin bones) is plotted against hip width (measured between the hooks) in the same logarithmic system, in which cranially located (external and internal) pelvic width measurements are independent variables, the tendency shown in Fig. 1 becomes apparent. In the light of previous research (Gere and Bartosiewicz, 1979*b*) this may be interpreted as follows: while the two external widths of the hindquarters show a harmonic growth marked by a slope almost parallel with the isometry line ($y = x$) of the coordinates, the widening of pelvic cavity is faster cranially, where the interpsoadic distance is measured. This is why it is impossible to obtain reliable data concerning the birth canal by exclusively gathering external measurements.

At the same time, this tendency increases the "bottleneck" caused by the bony ring formed by the sacral bone and pelvis at this end. In the region

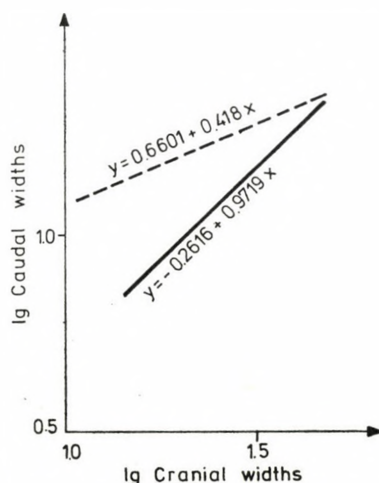

Fig. 1. **The allometric growth of external (continuous line) and internal (dashed line) pelvic widths. Dimensions measured on the cranial end of pelvis were used as independent variables. The convergence of slopes shows different growth intensities**

Table II

Comparison between the pelvic indices (and external hindquarter measurements) of beef-purpose (Limousine × Hungarian Fleckvieh) and dairy (Holstein Friesian) cows (all measurements in centimeters)

Beef cattle ($n = 78$)	$\bar{x}$	sd	cv	Pelvic index (%)		
				Minimum	Average	Maximum
Limousine × Red and White Hungarian F_1						
Cranial: hip width	52.9785	1.0401	0.0196	203.5666	204.2678	204.5967
Caudal: rump width	25.9358	1.0877	0.0419			
				Range = 1.0303%		
Dairy cattle ($n = 70$)						
Holstein Friesian						
Cranial: hip width	53.1251	1.0420	0.0196	197.4069	202.7683	208.1847
Caudal: rump width	26.1999	1.0919	0.0417			
				Range = 10.7780%		

of the more caudally located intertuberous distance soft tissues contribute more to the actual size of the birth canal.

The differential growth of internal and external rump measurements probably contributes to the fact that correlations between these two sets of dimensions are dynamically changing and are thus less useful in estimating calving properties (Holló et al., 1976). Cseh (1973), on the other hand, states that a 170 to 180 hip width to rump width percentage is ideal for easy calving. Experience also shows that judging external rump formation may be widely used in predicting calving ease: the wider the rump the easier the delivery of the calf (HFAA, 1982).

Table II contains univariate statistics for the external raw data plotted in Fig. 1. A sample of 70 dairy Holstein Friesian cows is presented as a parallel to emphasize the greater variability in comparison with the homogeneous F_1 generation of a beef-oriented crossing.

Although either of these breeds have special calving problems, pelvic indices fall consistently above 200%. The impressive homogeneity of the F_1 generation could be seen in the evaluation of internal pelvic measurements as well (Bartosiewicz and Gere, 1982). Thus, a very indirect, breed-specific relationship must be assumed between the external and internal measurements of the pelvic region, which may even be influenced by changing uniformity in the various generations of the crossing (Fábián, 1969).

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BOOK REVIEW

HÁMORI, D.: Constitutional Disorders and Hereditary Diseases in Domestic Animals

Akadémiai Kiadó, Budapest — Elsevier Scientific Publishing Company, Amsterdam, 1983. 728 pp.

The development of modern livestock breeding calls for a wide application of the most up-to-date selection methods and breeding techniques. That is why the progeny testing, the planning and organization of breeding providing a great selection pressure, as well as in certain domestic animal species a reasonably applied inbreeding and line breeding play an ever increasing role. These methods of increasing the volume and economic efficiency of production are necessarily accompanied by the more frequent occurrence of the genes that form the genetic basis of the standard qualities that are expected to improve. At the same time, however, beside the genes predetermining the desired properties, a more frequent manifestation of harmful factors representing genetic abnormalities must be reckoned with. In this respect a particularly great danger may arise from the overfavorization of a sire by artificial insemination and deep-freezing of sperm, if it carries recessive genes of some genetic abnormality in a heterozygous state of genotype. The fact that in consequence of the rapid development of the technique of sperm preservation and biotechnics as a whole (incl. embryo transfer and deep-freezing), the hereditary properties of any highly-qualified male (and to some extent of female) can be propagated in time and space almost boundlessly, further increases the importance of disclosing and studying the genetic anomalies and exercising a systematic control over them. The prevention of these losses is the joint task of animal breeders and veterinary surgeons specially trained in the genetics of domestic animals. The organs and institutions of breeding management have a very great responsibility in this field, with special regard to the international integration of breeding work (exchange of breeding animals and deep-frozen sperm as well as embryos, establishment of sperm banks etc.), and exploration and utilization of gene resources.

From the above-outlined situation it is clear that the Author — who was entitled by his wide knowledge of the special literature, several decades of practical experiences and experimental investigations to write this work (published as his posthumous writing) — undertook an extremely important, timely and involved task when preparing the manuscript of this comprehensive and pioneering work of the Hungarian special literature which is also of international interest.

The book runs to 728 pages with a large number (307) of illustrations, and is divided into the following main chapters:

I. General considerations (importance of heredopathology in animal breeding and production; the mode of inheritance of performance-limiting characters; importance of the elimination of undesirable qualities and the role of breeding hygiene in selection; the frequency of developmental disorders and pathological conditions; lethal defects and diseases; hereditary predisposition to diseases; prevention, identification and elimination of hereditary constitutional disorders; the role of artificial insemination in preventing the spread of abnormalities; the scope of breeding hygiene in large-scale production systems; the heredopathological importance of inbreeding; the impact of inbreeding and crossbreeding on cattle production; the impact of inbreeding on large-scale swine production; breeding hygiene in line- and crossbreeding of swine; the effect of inbreeding on the performance of the horse; the impact of inbreeding depression and crossbreeding

in other domestic animal species; immunological aspects of heredopathology in domestic animals).

II. Cytogenetic and clinical aspects of constitutional abnormality (cytogenetic study of abnormalities in body constitution; types of intersexuality; intersexual development in domestic animals; diagnosis of intersexuality; testicular feminization).

III. Fertility and prolificity (breeding hygiene problems in twinning; disorders of reproductive efficiency in sires; cryptorchidism; hereditary abnormalities of the male germ cell).

IV. Hereditary reproduction disorders in female domestic animals (the role of genetic prevention in sterility control; heritability of certain fertility factors; differences in fertility in large dairy units; familial correlations between life-span and fertility; hereditary factors responsible for pre- and postnatal losses in cattle; chromosome aberrations responsible for early fetal death; constitutional characteristics of fertility and vigour in breeding horses; constitutional differences in sow prolificity; perinatal losses in sheep, goat, poultry and rabbits).

V. Hereditary abnormalities in metabolism (metabolic diseases; the enzyme-diagnostic approach to metabolic disorders; metabolic disorders in various animal species; dwarfism, nanosomia; hydrops congenitus universalis; myodegeneration).

VI. Heredopathology of organs and organ systems (constitutional disorders of skin and hair; anomalies of the head, neck, vertebral column and central nervous system; conditions responsible for ataxia; hereditary anomalies of the eye).

VII. The gastrointestinal tract (anomalies of the gastrointestinal tract; inheritance of predisposition to hernia).

VIII. Respiratory diseases.

IX. The circulation system (developmental abnormalities; hereditary anomalies of the circulation system and haemopoiesis).

X. Diseases of the genitourinary system (hereditary renal diseases and developmental disorders).

XI. The lactiferous gland (breeding hygiene measures for the prevention of mastitis; aspects of judging the suitability of the udder for machine milking; relation of physiological parameters of the udder to mastitis; predisposition and resistance to mastitis in cow families; heredopathology of milk production in species other than the bovine species).

XII. The organs of motion (hereditary anomalies of the organs of motion; phokomelia and peromelia; acroteriasis congenita; posterior paralysis; crooked legs and other complex conditions; congenital splayleg syndrome; thick legs in domestic animals; osteofibrosis and osteoporosis; myotonia and other muscle diseases; anomalies of the extremities in poultry; the main motor disturbances in dogs; muscular hypertrophy; bone weakness syndrome; congenital subluxation of the patella; the spastic syndrome; interdigital overgrowth in cattle; quality and anomalies of the horny structures).

XIII. Resistance to diseases (genetically determined differences in resistance to infectious disease; resistance to parasites; utilization of disease resistance for the genetic prevention of diseases; the role of viruses in heredopathology).

XIV. Problems of multifactorial diseases and genetic resistance (atrophic rhinitis in swine; the heredopathology of neoplastic diseases; bovine leucosis and leucosis in other species)

XV. Appendix (recording of anomalies; administrative and herdbook measures).

The subchapters are generally completed by detailed references. The work is completed by author and subject index.

This book under review must be regarded as a pioneer undertaking in the Hungarian literature on livestock breeding and veterinary science. Hámori's work certainly excites great interest and gives rise to discussions in the special circles. The expected discussions can by all means be useful and may promote an organized protection against genetic abnormalities.

For the attractive make-up of the book the Publishing and Printing House of the Hungarian Academy of Sciences deserves praise.

J. Dohy

NEWS

8th Congress of the International Pig Veterinary Society 1984

The 8th International Congress of the International Pig Veterinary Society (I. P. V. S.) will be held from August 27–August 31, 1984 on the University Campus of the State University of Ghent, Belgium.

The scientific programme will last 3 days and will cover the following topics:

— Pig diseases and reproductive disorders: aetiology, pathogenesis, clinical aspects, pathology, immunology, prevention and treatment
— Pig nutrition
— Pig housing and management
— Health maintenance
— Miscellaneous

The scientific programme will be followed by visits to pig farms.

Information on the scientific programme, submission of papers etc. may be obtained from the organizing committee. Address: Faculty of Veterinary Medicine, Casinoplein 24, B-9000 Ghent, Belgium.

9th World Congress of the World's Small Animal Veterinary Association

The 9th World Congress of the World's Small Animal Veterinary Association (also 30th Annual Conference of the Expert Group on "Small Animal Diseases" of DVG — German Speaking Group of WSAVA) is being held from 19th to 22nd September 1984 in Hamburg, Federal Republic of Germany.

Applications for "Free Papers" should be sent as early as possible to: Dr. H. O. Schmidtke, President-Elect WSAVA, Hoffstrasse 6, D-7500 Karlsruhe, Federal Republic of Germany.

It is planned to have these papers presented as far as possible during the main program with simultaneous translation into German, English, French and Spanish.

5th Congress of the International Society for Animal Hygiene (first announcement)

The 5th Congress of the International Society for Animal Hygiene will be held from 10th to 13th September 1985 at the Veterinary School in Hannover, Federal Republic of Germany.

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Acknowledgement of grants and technical help.

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